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IDENTIFICATION OF ANTIMYCOBACTERIAL TARGETS AND THE INHIBITION THEREOF AS A TREATMENT FOR INFECTIOUS DISEASES

Cross Reference to Related Applications

[0001] The present application claims priority of Application Serial No. 60/420,131, filed 2002 October 22, which is hereby incorporated by reference in its entirety.

Government Support

[0002] This invention was made with U.S. Government support (NIH Grant No. AI43420) and the U.S. Government may therefore have certain rights in the invention.

Field of the Invention

[0003] The present invention is directed to the identification of anti-mycobacterial targets and the inhibition thereof as a treatment for infectious diseases, and more particularly, to the identification and inhibition of aerobic respiratory chains in mycobacterium as a treatment for infectious diseases. The invention is also directed to compositions comprising inactivated mycobacteria that can be used to induce immune responses in an individual.

Background of the Invention

[0004] Tuberculosis (TB) in all of its manifestations is the leading cause of death from a single infectious agent. Studies from two urban centers indicate that 30-40% of new cases are the result of recent infection rather than reactivation of old disease, and cases acquired by recent transmission accounted from almost two-third's of drug resistant TB.

[0005] Highly resistant strains of *M. tuberculosis* have been isolated from patients in the Philadelphia area at a rate that requires physicians to treat every new case of presumed TB with at least four drugs, i.e., to consider every new case as if it were caused by one of these resistant strains. Clearly, new approaches to the development of anti-tuberculosis therapy are necessary. However, the difficulties of working with *Mycobacterium tuberculosis* has kept the field from developing apace with the advances in molecular biology and biotechnology. In particular, the analysis of the regulation of DNA replication, traditionally a rich area for the discovery of new anti-microbial agents and one that would provide major new insights into the growth of Mycobacteria, has been slow to develop.

[0006] The complete biochemistry of mycobacteria is not fully understood which makes it difficult to combat and destroy harmful mycobacteria. Current attempts in treating the infectious diseases resulting from harmful mycobacteria (e.g. *Mycobacterium tuberculosis*), rely on destroying conventional targets within the mycobacteria, such as attacking the cell wall of the mycobacteria. Such practices however although may be therapeutic, however, are physically tasking to patients and are becoming increasingly marginalized as new and resistant strains of harmful mycobacteria are being discovered. Moreover, current practices do little to nothing to address the dormancy of harmful mycobacteria. Some harmful mycobacteria can exist, *in vivo*, in a dormant state waiting for more favorable environmental conditions to resurface and cause havoc.

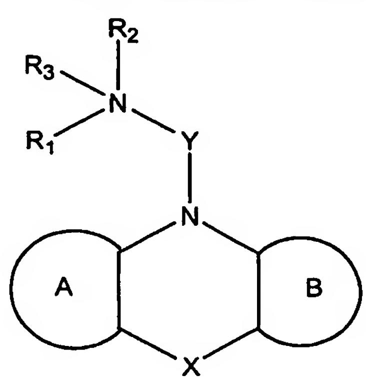
[0007] There is a need to identify and inhibit new antimycobacterial targets that will yield better results than currently inhibited or treated antimycobacterial targets. Thus, there is a need to identify and inhibit new antimycobacterial targets that will be more effective in combating and destroying mycobacteria that persist *in vivo* in a dormant state.

Summary of the Invention

[0008] The present invention provides methods of treating a patient with a *M. tuberculosis* infection comprising administering to the patient an amount of a composition comprising an electron transport chain inhibitor, wherein the amount is effective to modulate the electron transport chain in *M. tuberculosis* and does not have anti-dopaminergic effects in said patient.

[0009] In some embodiments the inhibitor is an oligonucleotide, a small molecule, a mimetic, a decoy, or an antibody.

[0010] In some embodiments the inhibitor is a small molecule of formula I,



I

wherein:

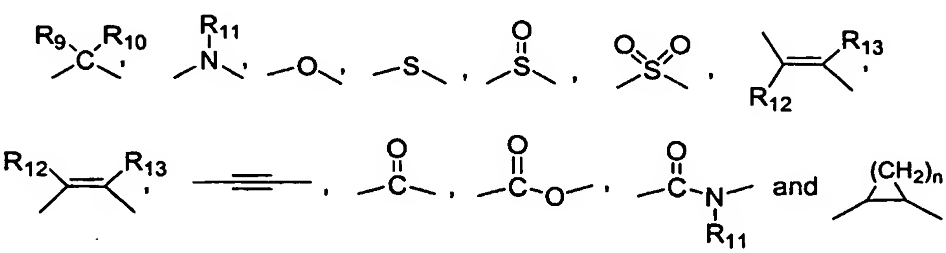
A and B are each independently aryl or heteroaryl and each are optionally substituted with 1-3 substituents selected from the group consisting of halogen, CHO, COR_4, C_1-C_6 alkyl, C_1-C_6 haloalkyl, C_1-C_6 hydroxyalkyl, C_1-C_6 alkenyl, C_1-C_6 alkynyl, C_1-C_6 alkoxy, phenyl (optionally substituted with 1-3 substituents selected from halogen, C_1-C_3 alkyl, C_1-C_3 haloalkyl, C_1-C_3 alkoxy, cyano, nitro, COOH, and CO_2R_4), heteroaryl (optionally substituted with 1-3 substituents selected from halogen, C_1-C_3 alkyl, C_1-C_3 haloalkyl, C_1-C_3 alkoxy, cyano, nitro, COOH, CO_2R_4), cyano, nitro, C_1-C_6 thioalkyl, C_1-C_6 thiohaloalkyl, C_1-C_6 alkylthiol, $(CH_2)_nCOOH$, $(CH_2)_nCO_2R_4$, $(CH_2)_nNR_5R_6$, $(CH_2)_nCONR_5R_6$, OH, SH, $(CH_2)_nNR_7COR_8$, $(CH_2)_nSOR_4$, SO_2R_4, $(CH_2)_nSONR_5R_6$, and $(CH_2)_nSO_2NR_5R_6$; and

n is an integer, wherein each n is independently selected from 0 to 6; and

R_4-R_8 are each independently selected from the group consisting of hydrogen, C_1-C_6 alkyl, and phenyl (optionally substituted with from 1-3 substituents selected from halogen, C_1-C_3 alkyl, C_1-C_3 haloalkyl, C_1-C_3 alkoxy, cyano, nitro, COOH, CO_2Me); or

R_5 and R_6 together with the nitrogen they are attached form a 5 to 7 member ring; and

Y is a linker unit consisting of 1 to 6 atoms or atom groups wherein the atom or atom groups are selected from the following:



and R_9 through R_{13} are each independently selected from a group consisting of hydrogen, C_1-C_6 alkyl, C_1-C_6 haloalkyl, and phenyl (optionally substituted with from 1-3 substituents selected from halogen, C_1-C_3 alkyl, C_1-C_3 haloalkyl, C_1-C_3 alkoxy, cyano, nitro, COOH, CO_2Me); and

R_1 and R_2 are each independently hydrogen, C_1-C_6 alkyl, $(CH_2)_nNR_4R_5$, phenyl (optionally substituted with 1-3 substituents selected from halogen, C_1-C_3 alkyl, C_1-C_3 haloalkyl, C_1-C_3 alkoxy, cyano, nitro, COOH, CO_2Me), CO_2R_4, $CO_2(CH_2)_n$phenyl (optionally substituted with from 1-3 substituents selected from halogen, C_1-C_3 alkyl, C_1-C_3 haloalkyl, C_1-C_3 alkoxy, cyano, nitro, COOH, CO_2Me), C_1-C_6 haloalkyl, cycloalkyl (optionally substituted with from 1-3 substituents selected from NR_5R_6, halogen, and C_1-C_6 alkyl), heterocycloalkyl including 1-3 hetero ring atoms selected from NR_{11}, O and S, optionally

substituted with 1-3 substituents selected from the group consisting of C_1-C_6 alkyl, C_1-C_6 hydroxyalkyl, C_1-C_6 haloalkyl, NO_2, CN, and $(CH_2)_nNR_4R_5$ or

R_1 and Y together with the nitrogen that they are attached form a 3 to 7 member ring; or

R_1 or R_2 together with the nitrogen that they are attached form a 3-7 member ring optionally containing from 1 to 3 additional heteroatoms selected from the group consisting of NR_{14}, O, and S, and optionally substituted with 1-3 substituents selected from the group consisting of C_1-C_6 alkyl, C_1-C_6 haloalkyl, NO_2, CN, and $(CH_2)_nNR_4R_5$; and

R_{14} is C_1-C_6 alkyl, C_1-C_6 haloalkyl, C_1-C_6 hydroxyalkyl, SO_2R_4, $SO_2NR_5R_6$, $CO(CH_2)_n$phenyl (optionally substituted with 1-3 substituents selected from C_1-C_6 alkyl, NR_5R_6, NO_2), $(CH_2)_n$phenyl (optionally substituted with 1-3 substituents selected from C_1-C_6 alkyl, halogen, NH_2, OH, OR, NO_2), CO_2R_4; and

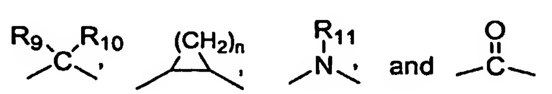
X is NR_{11}, O, S, SO, or SO_2; and

when both R_1 and R_2 are not hydrogen, R_3 is optionally present as H, C_1-C_6 alkyl, $(CH_2)_n$phenyl (optionally substituted with 1-3 groups selected from the group consisting of C_1-C_6 alkyl, halogen, NO_2, cyano, COOH, CO_2Me), or C_1-C_6 haloalkyl.

[0011] In some embodiments in the compound of formula I:

A and B are each aryl independently and optionally substituted with 1-3 substituents selected from the group consisting of halogen, C_1-C_3 haloalkyl, C_1-C_3 thioalkyl, C_1-C_3 thiohaloalkyl, cyano, $SO_2NR_5R_6$, SO_2R_4 and C_1-C_6 alkoxy; and

Y is a linker unit consisting of 1 to 4 atoms or atom groups wherein the atom or atom group is selected from the following



[0012] In some embodiments each aryl is independently naphthyl or phenyl. In some embodiments A and B are each phenyl and each phenyl is independently and optionally substituted with one substituent selected from halogen, CF_3, SMe, SCF_3, cyano, $SO_2N(Me)_2$, OMe, and SO_2Me; and X is S or SO_2. In some embodiemnts A is unsubstituted and B is substituted with Cl at the position para to X. In some embodiments A is unsubstituted and B is substituted with CF_3 at the position para to X. In some embodiments A is unsubstituted and B is substituted with SMe at the position para to X. In some embodiments A is unsubstituted

and B is substituted with SCF_3 at the position para to X. In some embodiments A is unsubstituted and B is substituted with $SO_2N(Me)_2$ at the position para to X. In some embodiments A is unsubstituted and B is substituted with OMe at the position para to X. In some embodiments A is unsubstituted and B is substituted with cyano at the position para to X. In some embodiments A is unsubstituted and B is substituted with SO_2Me at the position para to X. In some embodiments Y is $(CH_2)_3$; and R_1 and R_2 together form a 6-member ring with NR_{11} at the 4-position of the 6-member ring. In some embodiments Y is $(CH_2)_3$; and R_1, R_2 and R_3 are each methyl. In some embodiments Y is $(CH_2)_3$; and R_1 is benzyl, R_2 is methyl and R_3 is methyl.

[0013] The present invention also provides methods of treating a patient with a *M. tuberculosis* infection comprising administering to the patient an amount of a composition comprising a first inhibitor in combination with a second inhibitor, wherein the first inhibitor is administered in amounts effective to inhibit an electron transport system in *M. tuberculosis* but wherein the amount is not effective as an anti-dopaminergic in said patient, and wherein the second inhibitor is a traditional anti-tuberculosis medicament. In some embodiments the second inhibitor is isoniazid, rifampin, streptomycin, pyrazinamide or ethambutol.

[0014] The present invention further provides methods of modulating Type II NADH dehydrogenase in *M. tuberculosis* comprising contacting the *M. tuberculosis* with an amount of a composition comprising a *M. tuberculosis* modulator effective to modulate an electron transport chain in *M. tuberculosis* by at least 50%.

[0015] The present invention also provides methods of protecting an animal from a *M. tuberculosis* infection comprising administering to the animal an amount of a composition comprising a *M. tuberculosis* electron transport chain polypeptide modulator effective to modulate the electron transport chain in *M. tuberculosis.*

[0016] The present invention further provides methods of modulating respiration in a pathogen comprising administering to said pathogen an amount of a composition comprising a modulator effective to modulate the electron transport chain in *M. tuberculosis.*

[0017] The present invention also provides methods of modulating replication in a pathogen comprising administering to the pathogen an amount of a composition comprising a modulator effective to modulate the electron transport chain in the pathogen.

[0018] The present invention also provides methods of modulating growth of a pathogen comprising administering to the pathogen an amount of a composition comprising an modulator effective to effective to modulate the electron transport chain in the pathogen.

[0019] In some embodiments modulation of the electron transport chain is detected by measuring one or more of: oxidation of NADH, growth inhibition of *M. tuberculosis* or *M. smegmatis*, inhibition of respiration of *M. tuberculosis* or *M. smegmatis*, or inhibition of replication of *M. tuberculosis* or *M. smegmatis*. In some embodiments the electron transport chain is modulated by inhibiting one or more of type II NADH dehydrogenase, menaquinone, flavin adenine dinucleotide, bc1 complex, cytochrome bd oxidase, fumarate reductase, or nitrate reductase.

[0020] In some embodiments the effective amount inhibits the electron transport system in *M. tuberculosis* but does not have extrapyramidal side effects in said patient and/or does not block dopamine receptors in the patient. In some embodiments the inhibitor exhibits reduced side effects compared to treatment with a composition having anti-dopaminergic effects. In some embodiments the patient has not been diagnosed as having one or more psychological diseases or disorders at the time of treatment. In some embodiments the patient is not classified as psychotic according to the criteria of DSM-IV. In some embodiments the inhibitor is not chlorpromazine or trifluperazine. In some embodiments the *M. tuberculosis* is resistant to one or more of isoniazid, rifampin, streptomycin, pyrazinamide and ethambutol.

[0021] In some embodiments the inhibitor is an antibody selective for a *M. tuberculosis* electron transport chain polypeptide. In some embodiments the *M. tuberculosis* electron transport chain polypeptide has an amino acid sequence of SEQ ID NO:1, 3, 5, 7, 9 or 11.

[0022] In some embodiments the inhibitor is an antisense oligonucleotide comprising at least 80% sequence homology to the complement of a nucleic acid molecule encoding a *M. tuberculosis* electron transport chain polypeptide (SEQ ID NO:2, 4, 6, 8, 10 or 12), wherein said antisense oligonucleotide specifically hybridizes to the nucleic acid molecule and inhibits *M. tuberculosis* electron transport chain polypeptide mRNA levels by at least 50% in *M. tuberculosis*.

[0023] In some embodiments the inhibitor is an isolated polypeptide comprising a fragment of an electron transport chain polypeptide, said fragment at least 10 amino acid residues and comprising at least one epitope of the electron transport chain polypeptide.

[0024] In some embodiments the inhibitor is an isolated anti-electron transport chain polypeptide antibody obtained by immunization of a subject with an epitope-bearing fragment of an electron transport chain polypeptide.

[0025] The present invention also provides methods of treating a tuberculosis patient comprising administering to the patient a therapeutically effective amount of a composition of the invention. In some embodiments the composition comprises a live attenuated pathogen comprising a nucleotide sequence that encodes one or more *M. tuberculosis* electron transport chain polypeptides or functional fragments thereof. In some embodiments the composition comprises a recombinant vaccine comprising a nucleotide sequence that encodes one or more *M. tuberculosis* electron transport chain polypeptides or functional fragments thereof.

[0026] The present invention also provides methods for detecting the presence of *M. tuberculosis* in a sample. The methods comprise contacting the sample with an electron transport chain inhibitor comprising a detectable label and detecting evidence of the electron transport chain inhibitor in said sample, wherein evidence of the electron transport chain inhibitor is indicative of the presence of *M. tuberculosis*.

Brief Description of the Drawings

[0027] Figure 1 depicts proposed pathway of aerobic electron flow in mycobacteria. Complexes are illustrated as closed boxes with corresponding gene names and accession numbers written on the side.

[0028] Figures 2A, 2B and 2C: Figure 2A depicts the reduced and the carbon monoxide binding pigments of H37Rv cell extracts. (A) NADH-reduced minus air-oxidized difference spectrum. Reduction was accomplished 5 minutes after the addition of 10 mM NADH. (B) [NADH reduced plus carbon monoxide] minus [NADH reduced] difference spectrum. To illustrate the spectral perturbation due to carbon monoxide binding, 100 μM CO was added prior to reduction. Samples were suspended in 50 mM phosphate buffer (pH 7.4) at a protein concentration of 21.3 mg/ mL. Figure 2B depicts Trifluoperazine (TPZ) inhibition of NADH dependent oxygen consumption by *MTb* membranes. *MTb* membrane protein was added to phosphate buffer in a 2.0 ml vessel equipped with a Clark-type oxygen electrode. Respiration was initiated by the addition of 10 mM NADH, and arrrested upon the addition of 1 mM TPZ. Addition of 10 mM ascorbate and 1 mM TMPD produced an immediate resumption of respiration. Figure 2C depicts NADH reduced minus air oxidized *MTb* membranes in the

absence (solid line) or presence of 100 uM TPZ (dotted and hashed lines). The time in minutes after the addition of NADH is noted next to the trace. For each trace, 10 mM NADH was added to a 1 ml quartz semi-micro cuvette. For the samples containing TPZ, drug was added to the sample and incubated for 5 minutes before the addition of NADH.

[0029] Figures 3A and 3B: Figure 3A depicts titration of NADH:menaquinone oxidoreductase activity in the presence of Compound 1 (open boxes) or DMSO solvent (closed boxes). *M. tuberculosis* membrane protein was dialysed overnight in phosphate buffer to remove endogenous reducing agents depicts. The transfer of electrons from NADH to menaquinone-1 (MQ_1) was determined by monitoring the oxidation of NADH at 340 nm. NADH was not oxidized in the absence of membrane protein or MQ_1. Figure 3B depicts structures of analogs of phenothiazine.

[0030] Figure 4 depicts growth of *M. Smegmatis* OD_{600} after 48 hours in the presence of various inhibitors. Results are average of 6 independent experiments.

[0031] Figure 5 depicts growth of *Mycobacterium smegmatis* in the presence of various concentrations of chlorpromazine. Growth was measured by OD_{600} following 96 hours of incubation at 37°C while shaking at 150 RPM, and are average of 6 independent experiments.

[0032] Figures 6A and 6B: Figure 6A depicts oxygen uptake by *M. smegmatis* (50 mg/ml) after addition of phosphate buffer. Figure 6B depicts oxygen uptake by *M. smegmatis* (50 mg/ml) after addition of 1 mM chlorpromazine (B).

[0033] Figures 7A and 7B: Figure 7A depicts SDS-PAGE and Western Blot Analysis of Ndh and Schematic of Oxidation Assay. Figure 7B depicts purification scheme of NADH Type II reductase.

[0034] Figure 8: Activity of Purified Ndh protein. (A) Oxidation of NADH (B) Oxidation of NADH in the presence Quinone (C) Oxidation of NADH in the presence of FAD.

Detailed Description

[0035] *Mycobacterium tuberculosis* (*MTb*) infects more than one third of the world's population, causes active disease in more than 16 million people and accounts for over 3 million deaths annually. The extent of the problem will multiply with the spread of AIDS, with the emergence of multi-drug resistant strains and with insufficient public health measures in widespread areas of the world. Tuberculosis incidence as well as the development of multidrug-resistant tuberculosis has escalated due to the increasing

prevalence of HIV infection, increasing rates of homelessness and incarceration, and limited capacity of tuberculosis control programs to treat patients until cured (*2*). Although the disease can be treated with anti-microbial agents with the great majority of tubercle bacilli in a lesion killed within a week or two, it is necessary to continue multidrug therapy for at least five months to maximize the elimination of the few remaining bacilli. It is presumed that "persistors" lie dormant in an environment characterized by nutrient deprivation and oxygen limitation (*3*). It is also suggested that *MTb* can persist in this environment by entering a state of dormancy via the process of rapidly reducing or completely switching off protein synthesis in order to achieve a shutdown of cellular metabolic activity.

[0036] New approaches to the development of therapeutic agents for TB have been called for at the national and international levels (Nature 403, 687-692, 2000). The present inventors have identified two lead phenothiazine compounds, chlorpromazine and trifluperazine, both of which are FDA approved anti-psychotic agents. Our data indicates that these compounds inhibit mycobacterial growth and arrest cellular respiration. Spectrophotometric analysis identified the target of chlorpromazine action as type II NADH:menaquinone oxidoreducatse. There is no evidence of a mitochondrial-like type I NADH:menaquinone oxidoreductase activity in mycobacteria under aerobic conditions.

[0037] Although it should be appreciated that although the present invention is described in the context of *Mycobacterium tuberculosis*, the inventive concepts described may be applied to various mycobacteria and other Gram positive organisms.

Mycobacterium tuberculosis **respiration and adaptation to stationary phase:**

[0038] *Mycobacterium tuberculosis* is characterized as an obligately aerobic organism capable of survival in low oxygen environments by entering a state of dormancy. This state of quiescence is far from being well understood, but progress by Wayne and coworkers (Wayne, 1996, reviewed in Wayne, 1994) indicate that *MTb* "shifts down" to a dormant state through defined stages. Abrupt transfer of aerated *MTb* cultures to anaerobic conditions result in the death of the organism with a half-life of 10 hours (Wayne, 1982), whereas gradual depletion of oxygen permits tolerance to anaerobiasis. A drug that rapidly inhibits respiration in *MTb* may be acutely lethal, and would perhaps disrupt the organism's exit from dormancy.

[0039] Phenothiazines were produced by Paul Charpentier in the mid-1940s in an attempt to find new antihistamines (Charpentier, 1947). Courvosier and coworkers (1953) discovered that a chlorinated ring form, chlorpromazine, showed pronounced sedative activity with low

toxicity. It was first used as a perioperative anxiolytic agent, but later found use as an agent for agitated psychiatric patients. Phenothiazine compounds have been proposed for use against tuberculosis. However, until the present invention, the mechanism by which this class of drugs exerts its tuberculocidal effect was not well understood. Importantly, we show herein that trifluoperazine and chlorpromazine (CPZ) exert tuberculocidal activity against *Mycobacterium tuberculosis* strains resistant to isoniazid, rifampin, streptomycin, pyrazinamide and ethambutol combined. We have demonstrated that trifluoperazine and chlorpromazine are synergystic with both isoniazid and rifampicin. Despite the promise of this class of agents, the *in vitro* concentration of chlorpromazine and trifluperazine required for bacteriocidal activity is more than 100 fold greater than the concentration of drug achieved *in vivo*. While these drugs may be of benefit to tuberculosis patients suffering from schizophrenia, there is need to develop potent and specific analogs.

[0040] The antimycobacterial action of phenothiazines is independent of the neuroleptic activity of these compounds, which involves blockade of dopamine receptors within the brain (Creese, 1985, Kristiansen and Vergmann, 1986, Wyatt, 1986). Mycobacteria are not known to contain dopamine receptors.

[0041] While it is possible that phenothiazines may have multiple sites of action, it is also conceivable that they may act upon a primary process, such as energy production, to which all anabolic pathways are dependent. It is an integral part of our proposal to study the pathway of electron transfer in mycobacteria to further define the target of inhibitor action.

Electron transport in mitochondria is significantly different than in Mycobacteria:

[0042] While both mitochondria and mycobacteria pass electrons from succinate or NADH to oxygen via carriers of increasing reduction potential, the respiratory chains are considerably different in 4 major regards: 1) In bacteria, unique respiratory pigments and carriers, including hemes *o* and *d*, are present which are not found in mitochondria; 2) bacteria may utilize compounds other than oxygen as terminal electron acceptors; 3) the respiratory chain in bacteria may be branched with multiple terminals; 4) the composition of the electron transport chain is highly dependent on the growth conditions in bacteria (White & Sinclair, 1971, Stouthmammer, 1976).

[0043] Mitochondria reside in a constant cellular environment, and utilize oxygen in an unbranched pathway (figure below) (Garrett & Grisham, 1995, Hatefi, 1985). In contrast, respiration in *Mycobacterium tuberculosis* is branched, additionally utilizes hemes *d* and *o*,

contains an integrated cytochrome c into the bc_1 complex, and employs alternate cofactors (Yassin et al., 1988; Cole et. al. 1998). These differences may be exploited for chemotherapeutic development.

[0044] Oxidative phosphorylation is the process by which aerobic organisms generate energy for growth and survival in the form of ATP. It is catalyzed by a system of enzymes collectively called the electron transport chain (ETC). Electrons are shuttled from metabolic intermediates to oxygen via the ETC, and ATP is generated in the process. We believe that the ETC of bacteria is sufficiently different than the human counterparts as to provide the opportunity for new antibiotic compounds. In general, bacterial (prokaryotic) respiratory chains are considerably different than our own (Eukaryotic) in 4 major regards: 1) In prokaryotes, unique respiratory pigments and electron carriers, such as menaquinone and hemes *o* and *d*, are present which are not found in mitochondria (the site of the ETC in Eukaryotes). 2) bacteria may utilize compounds other than oxygen (nitrogen) as terminal electron acceptors; 3) the respiratory chain in bacteria may be branched with multiple terminals; 4) the composition of the electron transport chain is highly dependent on the growth conditions in bacteria.

[0045] Our studies have focused on *Mycobacterium tuberculosis* (*MTb*), an obligately aerobic gram positive organism that causes the disease tuberculosis in humans. *MTb* has several key respiratory enzymes that are significantly different than the eukaryotic (human) counterparts, and in particular, an essential type II NADH dehydrogenase (an enzyme that is part of the ETC) that is common to prokaryotes but absent in mammalian cells. It is anticipated that the inhibition of type II NADH dehydrogenase would greatly attenuate the growth of an organism. For example, inactivating mutations in *Mycobacterium smegmatis* *ndh* lead to a thermosensitive lethal and auxotrophic phenotype. We have identified a new class of compounds, phenothiazines, which can inhibit type II NADH dehydrogenase and therefore form the basis of a powerful new class of antibiotics. (Note: *M. smegmatis* is a different species of Mycobacteria that is used as a model for *MTb* because it grows faster and is non-lethal, so we do not have to work on it in P3 biohazard room; *ndh* is part of a series of genes that code for the type II NADH dehydrogenase enzyme).

[0046] There are two types of NADH dehydrogenases, properly called NADH:quinone oxidoreductases (bottom left of diagram). Type I is the variety found within mitochondria (in mammals). It is a complicated enzyme with 13-14 subunits, FMN as a cofactor, 9 iron-sulfur

clusters and an ability to pump protons across the membrane. Type II, commonly found in microbes, is far simpler. It is a single subunit with only FAD (flavin adenine dinucleotide) as a cofactor, contains no iron-sulfur clusters and is not energy coupled. *MTb* contains one set of genes, the *nuo* genes, encoding a type I NADH:quinone oxidoreductase, as well as two genes, *ndh* and *ndhA*, encoding 2 distinct type II oxidoreductases (2 different proteins). In many organisms, including *MTb*, type II is important in the growth phase, whereas type I is important during the stationary phase.

[0047] All of the known inhibitors of type I NADH:quinone oxidoreductase activity (rotenone, piericidin A, pyridaben, etc.) have been shown to be ineffective against type II, and we have confirmed this in *MTb*. The only known inhibitor of type II, flavone, is a weak inhibitor used as a laboratory analytical tool. Even at high concentrations *in vitro*, it is known that flavone is only a partial inhibitor of type II, so it is anticipated that the organism can adapt and become resistant to this compound *in vivo*. We have also shown that flavone is a weak inhibitor of type II NADH dehydrogenases in *MTb*. In addition, flavone has never been used as an antimicrobial in humans. There have not been any primary articles or review articles recommending the use of type II NADH:quinone oxidoreductases as a target for antimicrobials. We expect there to be minimal mechanisms of resistance to inhibitors of type II in *MTb* since type I NADH:quinone oxidoreductases are not important in growth, and provide the only alternative path of respiration. Gram positive bacteria that utilize menaquinone as a respiratory carrier appear most sensitive to inhibitors of type II NADH:quinone oxidoreductases.

[0048] Other possible uses for the type II NADH:quinone oxidoreductase target include gene therapy in humans with defective NADH:quinone oxidoreductase activity. This would include not only the treatment of Leber's hereditary optic neuropathy, which has been traced to specific mitochondrial DNA mutations, but also in the treatment of Parkinson's disease and focal dystonia.

[0049] Although it is appreciated that although the inventive concepts described herein are directed to the use of phenothiazines that include the analogs chlorpromazine and trifluperazine to impact the activities of the electron transport chain of mycobacteria, the inventive concepts described herein are extended to the use of similar or like compounds having similar or like effects on the electron transport chain of mycobacteria. Specifically, it

is appreciated that other heterocyclyl and polycyclyl compounds and their analogs used alone or in combination could be used to effect the activities of the electron chain transport.

***Other antimicrobial targets on the electron transport pathway*:**

[0050] Menaquinone Synthesis: Quinones are small organic molecules that serve as mobile electron carriers in the electron transport chain. Several families of quinones are known to exist in plants, eukaryotes, and microbes. *MTb* synthesizes and utilizes demethylmenaquinone-8 (DMK-8), whereas humans use ubiquinone (coenzyme Q). The two compounds are significantly different in structure and reduction potential. Although not wishing to be bound by theory, we hypothesize that phenothiazines may interfere with menaquinone function in *MTb* (drug design). ***The bc_1 complex***: The bc1 complex transfers electrons from quinone to cytochrome c in an energy-coupled reaction. This is an essential step in aerobic respiration and has been identified as the target of the drug atovaquone for the treatment of malaria. *MTb* contains a highly unusual bc_1 complex with a di-heme cytochrome c_1 subunit. It is predicted that inhibition of this complex would preclude logarithmic growth.

[0051] ***Cytochrome bd oxidase***: This enzyme transfers reductive potential from reduced quinone to oxygen, thus creating water. The enzyme has a high affinity for oxygen and is required for survival under low oxygen conditions. We recently showed that the *d*-type oxidase is induced in mycobacteria under low oxygen conditions and is important for microaerobic growth. Survival under microaerobic conditions has long been used a model of latent TB infection. Since it is predicted that the organism could not form a persistent infection without micoraerobic growth, a cytochrome *bd* knockout may be a good candidate for vaccine development.

[0052] ***Fumarate reductase***: Fumarate reductase is a flavoprotein that transfers electrons from reduced quinone to fumarate. Encoded by the frdABCD genes, it is an enzyme essential for anaerobic respiration. Rather than using oxygen as an electron acceptor, this enzyme allows *MTb* to respire on fumarate at the cost of inefficient energy production. A second anaerobic respiratory enzyme of interest is nitrate reductase (narGHIJX), which allows the organism to transfer electrons to nitrate instead of oxygen. Elmination or inhibition of these targets would reduce the ability of the organism to form a persistent infection, and may be useful in the development of chemotherapeutics or vaccines.

Definitions

[0053] As used herein, "modulation" means either an increase (stimulation) or a decrease (inhibition) in the expression of a gene or in the amount, quality, or effect of a particular activity or protein. In some embodiments of the present invention, inhibition is the form of modulation of gene expression.

[0054] As used herein, the term "inhibit" refers to a reduction or decrease in an activity, quality or quantity, compared to a baseline. For example, in the context of the present invention, inhibition of viral replication refers to a decrease in viral replication as compared to baseline. In some embodiments there is a reduction of about 30%, about 40%, about 50%, about 60%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 99%, and about 100%. Those of ordinary skill in the art can readily determine whether or not viral replication has been inhibited and to what extent.

[0055] As used herein, the terms "modulator," "inhibitor," "stimulator," or "activator" refers to compounds that can either modulate, inhibit, stimulate, or activate a function or activity. In some embodiments, the compounds are antibodies, peptides, polypeptides, small molecular weight compounds, antisense compounds, or RNAi compounds. In some embodiments, the antisense compound is an antisense oligonucleotide.

[0056] As used herein, the term "selectively inhibit" refers to selective inhibition of *M. tuberculosis* relative to other biological targets. For example, in some embodiments, the inhibitors of the present invention selectively inhibit *M. tuberculosis* but does not inhibit the dopamine pathway. In some embodiments, the affinity of the inhibitors of the present invention is at least 10, at least 10^2, or at least 10^3-fold higher for an element of the electron transport chain of *M. tuberculosis* than for a dopamine receptor. In some embodiments, the selectivity is determined by comparing IC_{50}, EC_{50}, MIC, or the like, between *in vivo* or *in vitro* models of *M. tuberculosis* cell growth, electron transport, cell respiration, cell replication, and *in vivo* or *in vitro* models of the human dopamine pathway.

[0057] In some embodiments the inhibitors of the present invention inhibit at least one activity of a *M. tuberculosis* electron transport chain polypeptide. In some embodiments, the inhibitors reduce the activity of a *M. tuberculosis* electron transport chain polypeptide by at least 50%, 60%, 70%, 75%, 80%, 90%, 95% or 100%, compared to a control.

[0058] As used herein, the term "small molecule" refers to a molecule with a molecular weight that is less than about 9, about 8, about 7, about 6, about 5, about 4, about 3, about 2, about 1 kilodaltons.

[0059] As used herein, the term "about" refers to +/- 20%, +/- 15%, +/- 10%, or +/- 5% of a given value.

[0060] As used herein, the term "prophylactically effective amount" is meant an amount of an inhibitor of the present invention effective to yield the desired prophylactic response. The specific prophylactically effective amount will, obviously, vary with such factors as the physical condition of the patient, the type of mammal or animal being treated, the duration of the treatment, the nature of concurrent therapy (if any), and the specific formulations employed and the structure of the inhibitor or its derivatives.

[0061] As used herein, the term "pathogen" refers to an agent that causes disease. In some embodiments the pathogen is a living microorganism such as a bacteria, especially a mycobacteria.

[0062] As used herein "mycobacteria" refers to any bacteria that falls within the mycobacterial genus. In some embodiments the mycobacteria is *S. aureus*, *E faecalis I*, *H. influenzae*, *M catarrhalis 1502*, *S. pneumoniae*, *E. Coli*, *M. tuberculosis* or *M. smegmatis*. In some embodiments the mycobacteria is *M. tuberculosis* or *M. smegmatis*.

[0063] As used herein, "attenuated *M. tuberculosis*" refers to a live bacteria that is less pathogenic than its normal wild-type form. A bacteria can be attenuated by various means known to the art-skilled. In some embodiments, the attenuated *M. tuberculosis* comprises an inactivated gene of the *M. tuberculosis* electron transport chain.

[0064] As used herein, an inactivated "*M. tuberculosis* electron transport chain gene" refers to an electron transport chain gene that is either not expressed or whose normal expression is reduced compared to the non-attenuated bacteria. In some embodiments the expression of the inactivated gene is about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 100% less than the non-attenuated gene.

[0065] As used herein "combination therapy" means that the individual in need of treatment is given another medicament for the disease in conjunction with the inhibitors of the present invention. This combination therapy can be sequential therapy where the individual is treated first with one or more drugs and then the other, or two or more drugs are given simultaneously.

[0066] As used herein, the term "an individual suspected of having been exposed to one or more pathogens" refers to an individual who has not been diagnosed as being positive for one or more pathogens but who could possibly have been exposed to one or more pathogens due

to a recent high risk activity or activity that likely put them in contact with the pathogens. For example, an individual suspected of having been exposed to *M. tuberculosis* refers to an individual that has been in close proximity to an individual infected with *M. tuberculosis* or having had contact or exposure to samples comprising *M. tuberculosis*. Examples of such samples include, without limitation, laboratory or research samples or samples of blood, semen, bodily secretions, and the like from patients. The individual from which the pathogen originated may or may not have been tested for the presence and/or absence of the pathogen. The term "an individual suspected of having been exposed to one or more pathogens" also includes individuals who have been diagnosed as being positive for one pathogen but are also infected with at least one further pathogen.

[0067] As used herein, the term "sample" refers to biological material from a patient. The sample assayed by the present invention is not limited to any particular type. Samples include, as non-limiting examples, single cells, multiple cells, tissues, tumors, biological fluids, biological molecules, or supernatants or extracts of any of the foregoing. Examples include tissue removed for biopsy, tissue removed during resection, blood, urine, saliva, lymph tissue, lymph fluid, cerebrospinal fluid, mucous, and stool samples. The sample used will vary based on the assay format, the detection method and the nature of the tumors, tissues, cells or extracts to be assayed. Methods for preparing samples are well known in the art and can be readily adapted in order to obtain a sample that is compatible with the method utilized.

[0068] The terms "subject" or "patient" as used herein include any mammalian species. In some embodiments, the methods of the present invention are contemplated for the treatment of infectious diseases in mammals such as humans, as well as those mammals of importance due to being endangered, of economical importance and/or social importance to humans.

[0069] As used herein, the term "antibody" is meant to refer to complete, intact antibodies, and Fab, Fab', F(ab)2, and other fragments thereof. Complete, intact antibodies include monoclonal antibodies such as murine monoclonal antibodies, chimeric antibodies and humanized antibodies.

[0070] As used herein, the term "epitope" refers to an antigenic determinant of a polypeptide. In some embodiments an epitope may comprise 3 or more amino acids in a spatial conformation which is unique to the epitope. In some embodiments epitopes are linear or conformational epitopes. Generally an epitope consists of at least 4 such amino acids, and

more usually, consists of at least 8-10 such amino acids. Methods of determining the spatial conformation of amino acids are known in the art, and include, for example, x-ray crystallography and 2-dimensional nuclear magnetic resonance.

[0071] "Fragment" as used herein refers to fragment of a protein that is less than the full-length of the wild-type protein. For example, if a polypeptide comprises a fragment of electron transport chain protein, it cannot have the complete contiguous amino acid sequence of the electron transport chain protein. In some embodiments, the polypeptide comprising a fragment of an electron transport chain protein can also comprise regions of another protein that is not an the electron transport chain protein of which the fragment is a part. In some embodiments the protein fragment is functional. For example, a functional fragment may have hydrolytic activity, synthetic activity, or the combination of both. A fragment having no activity is referred to as a "non-functional" fragment.

[0072] As used herein, the term "region" is meant a physically contiguous portion of the primary structure of a biomolecule. In the case of proteins, a region is defined by a contiguous portion of the amino acid sequence of that protein. In the case of polynucleotides, a region is defined by a contiguous portion of the nucleotide sequence of that polynucleotide. Examples of polynucleotide regions include without limitation, the 5'UTR, the start codon region, an intron/exon region, the stop codon region, and the 3'UTR. For example, the term "start codon region" refers to a portion of a polynucleotide that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a start codon. Similarly, the term "stop codon region" refers to a portion of a polynucleotide that encompasses from about 25 to about 50 contiguous nucleotides in either direction from a stop codon.

[0073] As used herein, the phrase "homologous nucleotide sequence," or "homologous amino acid sequence," or variations thereof, refers to sequences characterized by a homology, at the nucleotide level or amino acid level, of at least the specified percentage. Homologous nucleotide sequences include those sequences coding for isoforms of proteins. Such isoforms can be expressed in different tissues of the same organism as a result of, for example, alternative splicing of RNA. Alternatively, isoforms can be encoded by different genes. Homologous nucleotide sequences include nucleotide sequences encoding for a protein of a species other than *M. tuberculosis*, including, but not limited to, other bacteria and mammals. Homologous amino acid sequences include those amino acid sequences which contain

conservative amino acid substitutions and which polypeptides have the same binding and/or activity. Percent homology can be determined by, for example, the Gap program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, Madison WI), using the default settings, which uses the algorithm of Smith and Waterman (Adv. Appl. Math., 1981, 2, 482-489, which is incorporated herein by reference in its entirety).

[0074] As used herein, the term "isolated" nucleic acid molecule refers to a nucleic acid molecule (DNA or RNA) that has been removed from its native environment. Examples of isolated nucleic acid molecules include, but are not limited to, recombinant DNA molecules contained in a vector, recombinant DNA molecules maintained in a heterologous host cell, partially or substantially purified nucleic acid molecules, and synthetic DNA or RNA molecules.

[0075] As used herein, the term "oligonucleotide" refers to a series of linked nucleotide residues which has a sufficient number of bases to be used in a polymerase chain reaction (PCR). This short sequence is based on (or designed from) a genomic or cDNA sequence and is used to amplify, confirm, or reveal the presence of an identical, similar or complementary DNA or RNA in a particular cell or tissue. Oligonucleotides comprise portions of a DNA sequence having at least about 10 nucleotides and as many as about 50 nucleotides, preferably about 15 to 30 nucleotides. They may be chemically synthesized and may be used as probes.

[0076] The term "preventing" refers to decreasing the probability that a patient contracts or develops pathogenic infection.

[0077] The term "treating" refers to having a therapeutic effect and at least partially alleviating or ameliorating an abnormal condition in the patient.

[0078] The term "administering" relates to a method of delivering a compound to a cell or tissue of a patient. For cells harbored within a patient, many techniques exist in the art to administer compounds, including (but not limited to) oral, parenteral, dermal, injection, and aerosol applications.

[0079] As used herein, the phrase "stringent hybridization conditions" or "stringent conditions" refers to conditions under which a probe, primer, or oligonucleotide will hybridize to its target sequence, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences

hybridize specifically at higher temperatures. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength, pH and nucleic acid concentration) at which 50% of the probes complementary to the target sequence hybridize to the target sequence at equilibrium. Since the target sequences are generally present in excess, at T_m, 50% of the probes are occupied at equilibrium. Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes, primers or oligonucleotides (e.g. 10 to 50 nucleotides) and at least about 60°C for longer probes, primers or oligonucleotides. Stringent conditions may also be achieved with the addition of destabilizing agents, such as formamide.

[0080] As used herein, the term "electron transport chain" or "ETC" refers to a series of redox reactions where ATP is broken down into ADP, producing a net gain of energy in the organism. Elements of the electron transport chain in pathogens including M. tuberculosis include, without limitation, type II NADH dehydrogenase, menaquinone, flavin adenine dinucleotide, bc1 complex, cytochrome bd oxidase, fumarate reductase, and nitrate reductase.

[0081] As used herein, the term "pharmaceutical solid dosage forms" refers to a final solid pharmaceutical product. The term "pharmaceutical solid dosage form" includes, but is not limited to, tablets, caplets, beads, and capsules (including both hard shell capsules and soft gelatin capsules).

[0082] As used herein, the term "pharmaceutical liquid dosage form" includes liquid forms in which the compounds and compositions of the present invention can be incorporated for administration orally or by injection, and include aqueous solutions, suitably flavored syrups, aqueous or oil suspensions, and flavored emulsions with edible oils such as cottonseed oil, sesame oil, coconut oil, or peanut oil, as well as elixirs and similar pharmaceutical vehicles.

[0083] As used herein, the term "anti-dopaminergic effects" refers to compositions that decrease the release of dopamine, block dopamine receptors, have activity as dopamine antagonists or have side-effects attributable to dopamine blockage. Methods for determining whether compositions have anti-dopaminergic effects are known to those skilled in the art. Further methods are discussed in Huff *et al.*, (J. Pharmacol. Exp. Ther., 232, 57-61 (1984)) and Post *et al.* (Nature, 256, 342-343 (1975)). In some embodiments anti-dopaminergic

effects are exemplified as extrapyramidal side effects. (see Nielsen *et al.*, Acta.Pharmacol. Toxicol. (Copenh), 33, 353-362 (1973)).

[0084] As used herein, the term "psychological diseases or disorders" refers to diseases and disorders relating to the nervous system as discussed in the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), published in 1994 by the American Psychiatric Association.

[0085] As used herein, the term "not classified as psychotic according to the criteria of DSM-IV", refers to the criteria set forth in the DSM-IV for diagnosing an individual as psychotic.

[0086] In some embodiments, anti-dopaminergic effects of inhibitors of the present invention exhibit less than about 50%, 60%, 75%, 80%, 90%, or 95% of the anti-dopaminergic effects of known anti-psychotic medicaments used in patients with tuberculosis infections.

[0087] The term "alkyl", employed alone, is defined herein as, unless otherwise stated, either a straight-chain or branched saturated hydrocarbon moiety. In some embodiments, the alkyl moiety contains 1 to 12, 1 to 10, 1 to 8, 1 to 6, or 1 to 4 carbon atoms. Examples of saturated hydrocarbon alkyl moieties include, but are not limited to, chemical groups such as methyl, ethyl, *n*-propyl, isopropyl, *n*-butyl, *tert*-butyl, isobutyl, *sec*-butyl; higher homologs such as *n*-pentyl, *n*-hexyl, *n*-heptyl, *n*-octyl, and the like.

[0088] The term "alkenyl" is as defined above for "alkyl" except that the alkyl group contains one or more sites of unsaturation resulting in at least one carbon-carbon double bond. Such double bonds maybe present in either the cis- or trans- configurations. Some examples include, $-C{=}C{-}H$, $-CH_2{-}C{=}CH$, $-C{=}C{-}CH_3$, $-(CH_2)_2{-}C{=}C{-}H$, $-(CH_2){-}C{=}C{-}CH_3$, and the like.

[0089] The term alkynyl is as defined above for "alkyl" except that the alkyl group contains one or more sites of double unsaturation resulting in at least one carbon-carbon triple bond. Some examples include $-\equiv-H$, $-\equiv-CH_3$, $-\equiv-CH_2CH_3$, $-(CH_2)-\equiv$, and the like.

[0090] The term "alkylenyl" refers to a bivalent straight-chained or branched alkyl group.

[0091] As used herein, "haloalkyl" refers to an alkyl group having one or more halogen substituents. Example haloalkyl groups include CF_3, C_2F_5, CHF_2, CH_2CF_3, CCl_3, $CHCl_2$, C_2Cl_5, and the like.

[0092] The term "alkoxy", employed alone or in combination with other terms, is defined herein as, unless otherwise stated, -O-alkyl. Examples of alkoxy moieties include, but are not

limited to, chemical groups such as methoxy, ethoxy, isopropoxy, *sec*-butoxy, *tert*-butoxy, and homologs, isomers, and the like.

[0093] The term "haloalkoxy", employed alone or in combination with other terms, is defined herein as, unless otherwise stated, -O-haloalkyl. Examples of alkoxy moieties include, but are not limited to, chemical groups such $-OCF_3$, $-OCH_2CH_2Cl$, and the like.

[0094] The term "cycloalkyl", employed alone or in combination with other terms, is defined herein as, unless otherwise stated, a monocyclic, bicyclic, tricyclic, fused, bridged, or spiro monovalent saturated hydrocarbon moiety of 3-8 or 3-7 carbon atoms. Any suitable ring position of the cycloalkyl moiety can be covalently linked to the defined chemical structure. Examples of cycloalkyl moieties include, but are not limited to, chemical groups such as cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, norbornyl, adamantyl, spiro[4.5]decanyl, and homologs, isomers, and the like.

[0095] As used herein, "heterocycloalkyl" refers to a cycloalkyl group wherein one or more of the ring-forming carbon atoms is replaced by a heteroatom such as an O, S, N, or P atom. Also included in the definition of heterocycloalkyl are moieties that have one or more aromatic rings fused (i.e., having a bond in common with) to the nonaromatic heterocyclic ring, for example phthalimidyl, naphthalimidyl pyromellitic diimidyl, phthalanyl, and benzo derivatives of saturated heterocycles such as indolene and isoindolene groups.

[0096] The terms "halo" or "halogen", employed alone or in combination with other terms, is defined herein as, unless otherwise stated, fluoro, chloro, bromo, or iodo.

[0097] The term "aryl", employed alone or in combination with other terms, is defined herein as, unless otherwise stated, an aromatic hydrocarbon of up to 14 carbon atoms, which can be a single ring (monocyclic) or multiple rings (bicyclic, up to three rings) fused together or linked covalently. Any suitable ring position of the aryl moiety can be covalently linked to the defined chemical structure. Examples of aryl moieties include, but are not limited to, chemical groups such as phenyl, naphthyl, 1-naphthyl, 2-naphthyl, dihydronaphthyl, tetrahydronaphthyl, biphenyl, anthryl, phenanthryl, fluorenyl, indanyl, biphenylenyl, acenaphthenyl, acenaphthylenyl, and the like.

[0098] The term "arylalkyl" or "aralkyl," employed alone or in combination with other terms, is defined herein as, unless otherwise stated, an alkyl, as herein before defined, substituted with an aryl moiety. Examples of arylalkyl moieties include, but are not limited to, chemical

groups such as benzyl, 1-phenylethyl, 2-phenylethyl, diphenylmethyl, 3-phenylpropyl, 2-phenylpropyl, fluorenylmethyl, and homologs, isomers, and the like.

[0099] As used herein, "heteroaryl" groups are monocyclic and polycyclic aromatic hydrocarbons that have at least one heteroatom ring member such as sulfur, oxygen, or nitrogen. Heteroaryl groups include, without limitation, pyridyl, pyrimidinyl, pyrazinyl, pyridazinyl, triazinyl, furyl, quinolyl, isoquinolyl, thienyl, imidazolyl, thiazolyl, indolyl, pyrryl, oxazolyl, benzofuryl, benzothienyl, benzthiazolyl, isoxazolyl, pyrazolyl, triazolyl, tetrazolyl, indazolyl, 1,2,4-thiadiazolyl, isothiazolyl, benzothienyl, purinyl, carbazolyl, benzimidazolyl, 2,3-dihydrobenzofuranyl, 2,3-dihydrobenzothienyl, 2,3-dihydrobenzothienyl-S-oxide, 2,3-dihydrobenzothienyl-S-dioxide, benzoxazolin-2-on-yl, indolinyl, benzodioxolanyl, benzodioxane, and the like. In some embodiments, heteroaryl groups can have from 1 to about 20 carbon atoms, and in further embodiments from about 3 to about 20 carbon atoms. In some embodiments, heteroaryl groups have 1 to about 4, 1 to about 3, or 1 to 2 heteroatoms.

[00100] As used herein, "heterocycle" refers to a heteroaryl or heterocycloalkyl group.

[00101] As used herein, the term "leaving group" refers to a moiety that can be displaced by another moiety, such as by nucleophilic attack, during a chemical reaction. Leaving groups are well known in the art and include, for example, halides and OSO_2-R' where R' is, for example, alkyl, haloalkyl, or aryl optionally substituted by halo, alkyl, alkoxy, amino, and the like. Some example leaving groups include chloro, bromo, iodo, mesylate, tosylate, and other similar groups.

[00102] As used herein, the term "reacting" refers to the bringing together of designated chemical reactants such that a chemical transformation takes place generating a compound different from any initially introduced into the system. Reacting can take place in the presence or absence of solvent.

[00103] As used herein, the term hydroxyalkyl refers to an alkyl group as described previously where one or more of the carbons of the alkyl group is substituted with a hydroxy group. In some cases, the hydroxy group may be esterified. Such esters may be prepared via reaction of one or more free hydroxy groups of the hydroxy alkyl chain and a C_1-C_{18} carboxylic acid or C_1-C_{18} acid halide. Some non-limiting examples are -CH2OH, -(CH2)2OH, $-C(CH_3)_2OH$, $-(CH_2)_4-OH$, $-CH(CH_3)CH_2OH$, $-(CH_2)_5-OH$, $-CH(CH_3)(CH_2)_4-OH$, $-CH_2-CH(OH)(CH_2)_2OH$, and the like.

[00104] As used herein, thioalkyl refers to a –S-alkyl group wherein alkyl is as defined above. Some non-limiting examples of thioalkyl are $-SCH_3$, $-SCH_2CH_3$, $S-CH(CH_3)_2$, $-S(CH_2)_3CH_3$, and the like.

[00105] As used herein, alkylthio refers to –alkyl-S-(H or alkyl) where alkyl is as defined above. Some non-limiting examples are $-CH_2SH$, $-CH_2CH_2SH$, $-CH_2SCH_2CH_3$, $-CH_2CH_2SCH_3$, $-CH_2SCH(CH_3)_2$ and the like.

[00106] As used herein, thiohaloalkyl refers to a –S-haloalkyl group where haloalkyl is as defined previously. Some non-limiting examples are $-S-CH_2CH_2Cl$, $-S-CH_2CH_2Br$, $S-(CH_2)_3Cl$, $S-CH\ (CH_2Cl)CH_3$ and the like.

Methods of treating tuberculosis in a patient

[00107] The present invention provides methods of treating a patient with a *M. tuberculosis* infection comprising administering to the patient an amount of a composition comprising an electron transport chain inhibitor, wherein said amount is effective to modulate the electron transport chain in said *M. tuberculosis* does not have anti-dopaminergic effects in said patient. In some embodiments the inhibitor is an oligonucleotide, a small molecule, a mimetic, a decoy, or an antibody.

[00108] In some embodiments a second inhibitor is administerd to the patient wherein the second inhibitor is isoniazid, rifampin, streptomycin, pyrazinamide or ethambutol. In some embodiments the first and second inhibitors each has an IC50 greater than 100 μM in a D2 dopamine receptor binding assay. In some embodiments the first and second inhibitors each has an IC50 greater than 300 μM in a D2 dopamine receptor binding assay. In some embodiments the first and second inhibitors each has an IC50 less than 100 μM in an electron transport system model. In some embodiments the first and second inhibitors each has an IC50 less than 30 μM in an electron transport system model. In some embodiments the first and second inhibitors each has an IC50 less than 30 μM in an electron transport system model and an IC50 greater than 100 μM in a D2 dopamine receptor binding assay.

[00109] The present invention also provides methods of modulating Type II NADH dehydrogenase in *M. tuberculosis* comprising contacting said cell with an amount of a composition comprising a *M. tuberculosis* modulator, said amount effective to modulate an electron transport chain in said *M. tuberculosis* by at least 50%.

[00110] The present invention also provides methods of protecting an animal from a *M. tuberculosis* infection comprising administering to said animal an amount of a composition

comprising a *M. tuberculosis* electron transport chain polypeptide modulator effective to modulate the electron transport chain in *M. tuberculosis.*

[00111] The present invention also provides methods of modulating respiration in a pathogen comprising administering to said pathogen an amount of a composition comprising a modulator effective to modulate the electron transport chain in *M. tuberculosis.*

[00112] The present invention also provides methods of modulating replication in a pathogen comprising administering to said pathogen an amount of a composition comprising a modulator effective to modulate the electron transport chain in *M. tuberculosis.*

[00113] The present invention also provides methods of modulating growth of a pathogen comprising administering to said pathogen an amount of a composition comprising an modulator effective to effective to modulate the electron transport chain in *M. tuberculosis.*

[00114] In some embodiments modulation of the electron transport chain is detected by measuring one or more of: oxidation of NADH, growth inhibition of *M. tuberculosis* or *M. smegmatis*, inhibition of respiration of *M. tuberculosis* or *M. smegmatis*, or inhibition of replication of *M. tuberculosis* or *M. smegmatis.* In some embodiments inhibition is at least 50% as compared to a control. In some embodiments the electron transport chain is modulated by inhibiting one or more type II NADH dehydrogenase, menaquinone, flavin adenine dinucleotide, bc1 complex, cytochrome bd oxidase, fumarate reductase, or nitrate reductase. In some embodiments the effective amount inhibits the electron transport system in *M. tuberculosis* but does not have extrapyramidal side effects in said patient. In some embodiments the effective amount inhibits the electron transport system in *M. tuberculosis* and does not block dopamine receptors. In some embodiments the inhibitor exhibits reduced side effects compared to treatment with a composition having anti-dopaminergic effects. In some embodiments the patient has not been diagnosed as having one or more psychological diseases or disorders at the time of treatment. In some embodiments the patient is not classified as psychotic according to the criteria of DSM-IV. In some embodiments the composition is effective at a concentration of less than about 10 μM to inhibit electron flow by at least 50% in an *in vitro* assay of electron transport. In some embodiments the inhibitor is not chlorpromazine or trifluperazine.

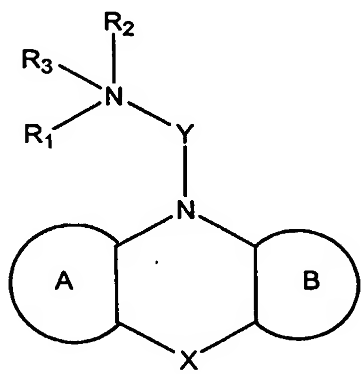
[00115] In some embodiments the inhibitor is an antibody selective for a *M. tuberculosis* electron transport chain polypeptide having an amino acid sequence of SEQ ID NO:1, 3, 5, 7, 9 or 11.

[00116] In some embodiments the *M. tuberculosis* is resistant to one or more of isoniazid, rifampin, streptomycin, pyrazinamide and ethambutol.

[00117] In some embodiments, the inhibitor of the present invention is not an composition discussed in one or more of Fischer et al., (Diseases of the Chest 34: 134-9, 1959); Bourdon (Annales de l'Institute Pasteur 101: 876-86, 1961); Dawkins et al., (Biochemical Journal 72: 204-9, 1958); Molnar et al., Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene I. Abterilung Originale A 239: 521-6, 1977); or Kristiansen et al., (Pharmacol Toxicol 60:100-103, 1987).

Small molecules

[00118] In some embodiments, the inhibitors are small molecules having formula I,



I

wherein:

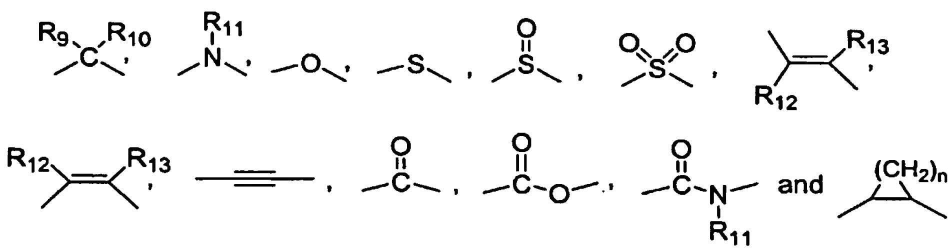
A and B are each independently aryl or heteroaryl and each are optionally substituted with 1-3 substituents selected from the group consisting of halogen, CHO, COR_4, C_1-C_6 alkyl, C_1-C_6 haloalkyl, C_1-C_6 hydroxyalkyl, C_1-C_6 alkenyl, C_1-C_6 alkynyl, C_1-C_6 alkoxy, phenyl (optionally substituted with 1-3 substituents selected from halogen, C_1-C_3 alkyl, C_1-C_3 haloalkyl, C_1-C_3 alkoxy, cyano, nitro, COOH, and CO_2R_4), heteroaryl (optionally substituted with 1-3 substituents selected from halogen, C_1-C_3 alkyl, C_1-C_3 haloalkyl, C_1-C_3 alkoxy, cyano, nitro, COOH, CO_2R_4), cyano, nitro, C_1-C_6 thioalkyl, C_1-C_6 thiohaloalkyl, C_1-C_6 alkylthiol, $(CH_2)_nCOOH$, $(CH_2)_nCO_2R_4$, $(CH_2)_nNR_5R_6$, $(CH_2)_nCONR_5R_6$, OH, SH, $(CH_2)_nNR_7COR_8$, $(CH_2)_nSOR_4$, SO_2R_4, $(CH_2)_nSONR_5R_6$, and $(CH_2)_nSO_2NR_5R_6$; and

n is an integer, wherein each n is independently selected from 0 to 6; and

R_4-R_8 are each independently selected from the group consisting of hydrogen, C_1-C_6 alkyl, and phenyl (optionally substituted with from 1-3 substituents selected from halogen, C_1-C_3 alkyl, C_1-C_3 haloalkyl, C_1-C_3 alkoxy, cyano, nitro, COOH, CO_2Me); or

R_5 and R_6 together with the nitrogen they are attached form a 5 to 7 member ring; and

Y is a linker unit consisting of 1 to 6 atoms or atom groups wherein the atom or atom groups are selected from the following:



R_9 through R_{13} are each independently selected from a group consisting of hydrogen, C_1-C_6 alkyl, C_1-C_6 haloalkyl, and phenyl (optionally substituted with from 1-3 substituents selected from halogen, C_1-C_3 alkyl, C_1-C_3 haloalkyl, C_1-C_3 alkoxy, cyano, nitro, COOH, CO_2Me); and

R_1 and R_2 are each independently hydrogen, C_1-C_6 alkyl, $(CH_2)_nNR_4R_5$, phenyl (optionally substituted with 1-3 substituents selected from halogen, C_1-C_3 alkyl, C_1-C_3 haloalkyl, C_1-C_3 alkoxy, cyano, nitro, COOH, CO_2Me), CO_2R_4, $(CH_2)_n$phenyl (optionally substituted with from 1-3 substituents selected from halogen, C_1-C_3 alkyl, C_1-C_3 haloalkyl, C_1-C_3 alkoxy, cyano, nitro, COOH, CO_2Me), $CO_2(CH_2)_n$phenyl (optionally substituted with from 1-3 substituents selected from halogen, C_1-C_3 alkyl, C_1-C_3 haloalkyl, C_1-C_3 alkoxy, cyano, nitro, COOH, CO_2Me), C_1-C_6 haloalkyl, cycloalkyl (optionally substituted with from 1-3 substituents selected from NR_5R_6, halogen, and C_1-C_6 alkyl), heterocycloalkyl including 1-3 hetero ring atoms selected from NR_{11}, O and S, optionally substituted with 1-3 substituents selected from the group consisting of C_1-C_6 alkyl, C_1-C_6 hydroxyalkyl, C_1-C_6 haloalkyl, NO_2, CN, and $(CH_2)_nNR_4R_5$; or

R_1 and Y together with the nitrogen that they are attached to form a 3 to 7 member ring; or

R_1 or R_2 together with the nitrogen that they are attached form a 3-7 member ring optionally containing from 1 to 3 additional heteroatoms selected from the group consisting

of NR_{14}, O, and S, and optionally substituted with 1-3 substituents selected from the group consisting of C_1-C_6 alkyl, C_1-C_6 hydroxyalkyl, C_1-C_6 haloalkyl, NO_2, CN, and $(CH_2)_nNR_4R_5$; and

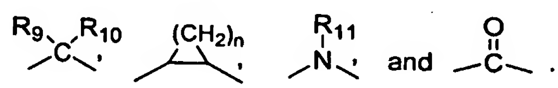
R_{14} is C_1-C_6 alkyl, C_1-C_6 haloalkyl, C_1-C_6 hydroxyalkyl, SO_2R_4, $SO_2NR_5R_6$, $CO(CH_2)_n$phenyl (phenyl optionally substituted with 1-3 substituents selected from C_1-C_6 alkyl, NR_5R_6, NO_2), $(CH_2)_n$phenyl (phenyl optionally substituted with 1-3 substituents selected from C_1-C_6 alkyl, halogen, NH_2, OH, OR, NO_2), CO_2R_4; and

X is NR_{11}, O, S, SO, or SO_2; and

when both R_1 and R_2 are not hydrogen, R_3 is optionally present as H, C_1-C_6 alkyl, $(CH_2)_n$phenyl (phenyl optionally substituted with 1-3 groups selected from the group consisting of C_1-C_6 alkyl, halogen, NO_2, cyano, COOH, CO_2Me), or C_1-C_6 haloalkyl.

[00119] In some embodiments, A and B are each aryl independently and optionally substituted with 1-3 substituents selected from the group consisting of halogen, C_1-C_3 haloalkyl, C_1-C_3 thioalkyl, C_1-C_3 thiohaloalkyl, cyano, $SO_2NR_5R_6$, SO_2R_4 and C_1-C_6 alkoxy; and

Y is a linker unit consisting of 1 to 4 atoms or atom groups wherein the atom or atom group is selected from the following:



[00120] In some embodiments, aryl is independently naphthyl or phenyl.

[00121] In some embodiments, A and B are each phenyl and each phenyl is independently and optionally substituted with one substituent selected from halogen, CF_3, SMe, SCF_3, cyano, $SO_2N(Me)_2$, OMe, and SO_2Me; and X is S or SO_2.

[00122] In some embodiments, A is unsubstituted phenyl and B is phenyl substituted with Cl at the position para to X.

[00123] In some embodiments, A is unsubstituted phenyl and B is phenyl substituted with CF_3 at the position para to X.

[00124] In some embodiments, A is unsubstituted phenyl and B is phenyl substituted with SMe at the position para to X.

[00125] In some embodiments, A is unsubstituted phenyl and B is phenyl substituted with SCF_3 at the position para to X.

[00126] In some embodiments, A is unsubstituted phenyl and B is phenyl substituted with $SO_2N(Me)_2$ at the position para to X.

[00127] In some embodiments, A is unsubstituted phenyl and B is phenyl substituted with OMe at the position para to X.

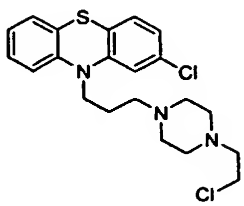
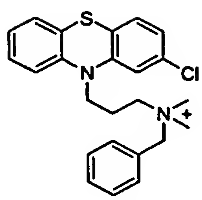
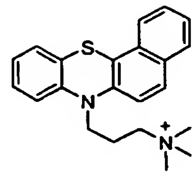
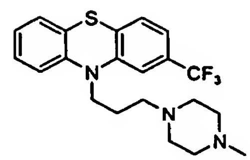
[00128] In some embodiments, A is unsubstituted phenyl and B is phenyl substituted with SO_2Me at the position para to X.

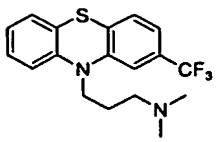
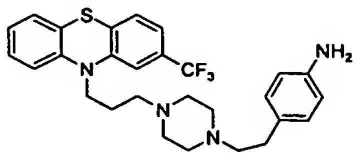
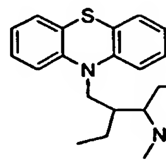
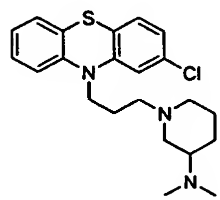
[00129] In some embodiments, Y is $(CH_2)_3$; and R_1 and R_2 together form a 6-member ring with NR_{11} at the 4-position of the 6-member ring.

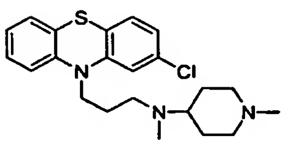
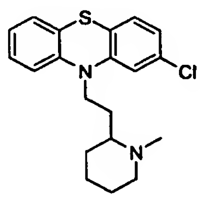
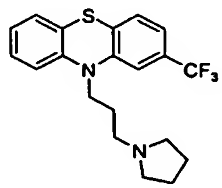
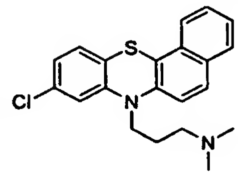
[00130] In some embodiments, Y is $(CH_2)_3$; and R_1, R_2 and R_3 are each methyl.

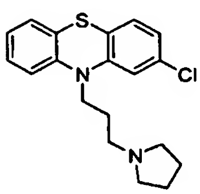
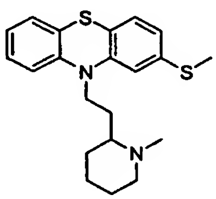
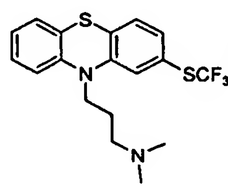
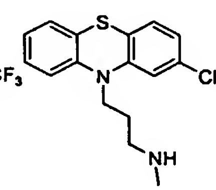
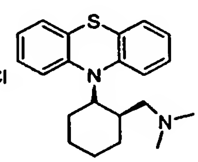
[00131] In some embodiments, Y is $(CH_2)_3$; and R_1 is benzyl, R_2 is methyl and R_3 is methyl.

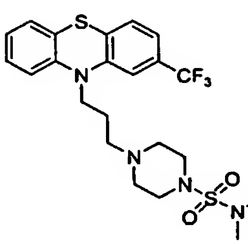
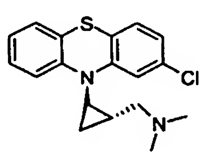
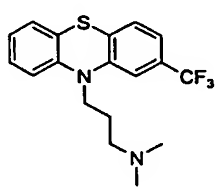
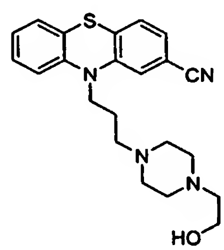
[00132] In some embodiments, one or more of the compound(s) of formula I is or is not:

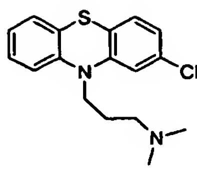
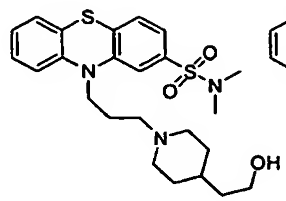
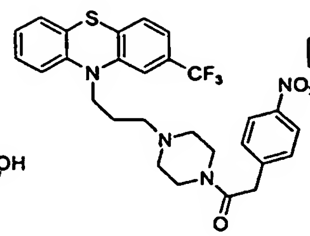
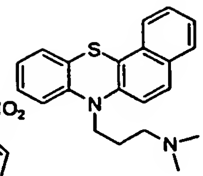
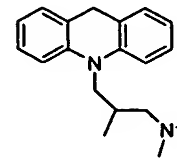
   

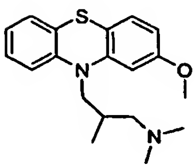
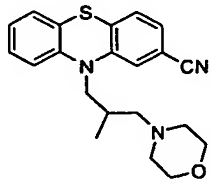
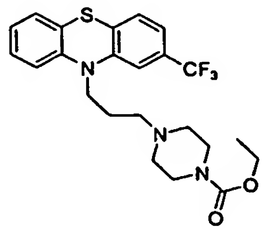
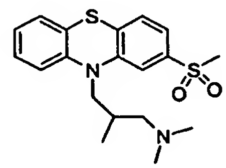
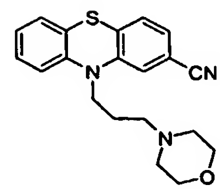
   

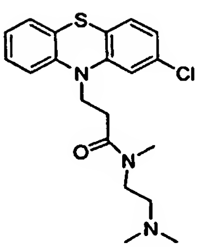
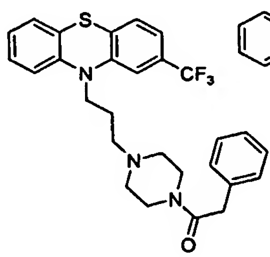
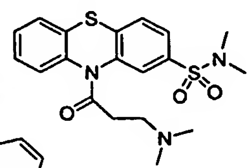
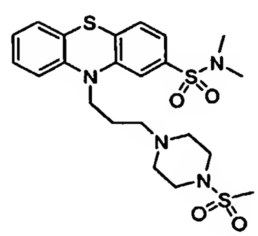
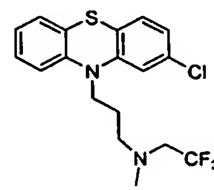
    

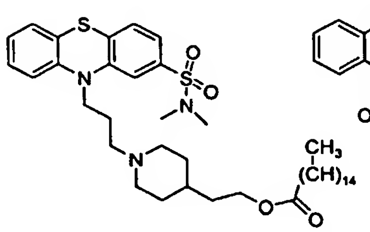
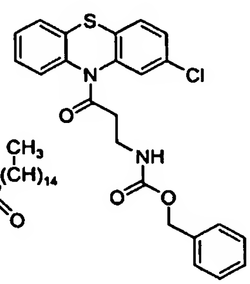
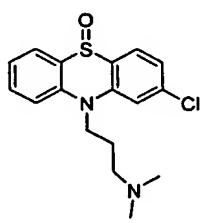
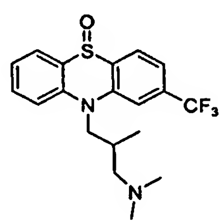
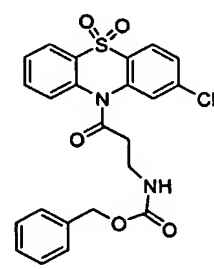
   

[00133] In some embodiments, one or more of the compound(s) of formula I is or is not:

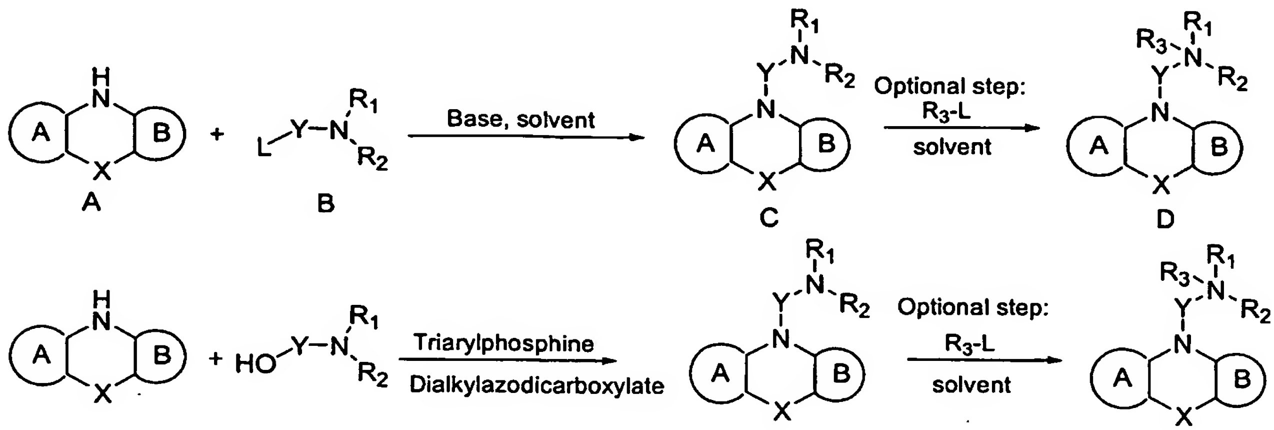






[00134] Methods for preparing compounds of the invention are described in the generic fashion illustrated below. The general method is comprised of the reaction of the tricyclic starting material A with the side chain linker moiety B in the presence of an appropriate base and solvent where L is a suitable leaving group. The product C formed as a result of the reaction between A and B maybe further reacted with an alkylating agent R_3-L to form the quaternary salt D.

[00135] Alternatively, a Mitsunobu-type reaction can be utilized wherein instead of a typical leaving group L, an alcohol (preferably a primary alcohol) is used instead. The

starting materials A and B are either commercially available or maybe prepared by methods well-known to one of ordinary skill in the art.



[00136] It is understood in the generic description above and for other groups described herein that, in each instance any variable group may be independently substituted by their allowed groups. Thus, for example, where a structure is described wherein two R_2 groups are simultaneously present on the same compound; the two R_2 groups can represent different groups.

[00137] It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, can also be provided in combination in a single embodiment. Conversely, various features of the invention which are, for brevity, described in the context of a single embodiment, can also be provided separately or in any suitable subcombination.

[00138] The compounds of the present invention can contain an asymmetric atom, and some of the compounds can contain one or more asymmetric atoms or centers, which can thus give rise to optical isomers (enantiomers) and diastereomers. The present invention includes such optical isomers (enantiomers) and diastereomers (geometric isomers); as well as the racemic and resolved, enantiomerically pure *R* and *S* stereoisomers; as well as other mixtures of the *R* and *S* stereoisomers and pharmaceutically acceptable salts thereof. Optical isomers can be obtained in pure form by standard procedures known to those skilled in the art, and include, but are not limited to, diastereomeric salt formation, kinetic resolution, and asymmetric synthesis. It is also understood that this invention encompasses all possible regioisomers, and mixtures thereof, which can be obtained in pure form by standard separation procedures known to those skilled in the art, and include, but are not limited to,

column chromatography, thin-layer chromatography, and high-performance liquid chromatography.

[00139] The compounds provided herein can also include their salts formed from, for example, mineral or organic acid salts of basic residues such as amines; alkali or organic salts of acidic residues such as carboxylic acids; and the like. The invention includes acceptable salt forms formed from the addition reaction with either inorganic or organic acids. Additionally, this invention includes quaternary ammonium salts of the compounds herein, which can be prepared by reacting the nucleophilic amines with a suitably reactive alkylating agent such as an alkyl halide or benzyl halide. Lists of suitable salts are found in *Remington's Pharmaceutical Sciences*, 17th ed., Mack Publishing Company, Easton, Pa., 1985, p. 1418, the disclosure of which is hereby incorporated by reference.

[00140] Compounds of the invention can also include all isotopes of atoms occurring in the intermediates or final compounds. Isotopes include those atoms having the same atomic number but different mass numbers. For example, isotopes of hydrogen include tritium and deuterium.

[00141] Compounds of the invention can also include tautomeric forms, such as keto-enol tautomers. Tautomeric forms can be in equilibrium or sterically locked into one form by appropriate substitution.

[00142] The processes described herein can be monitored according to any suitable method known in the art. For example, product formation can be monitored by spectroscopic means, such as nuclear magnetic resonance spectroscopy (e.g., ^{1}H or ^{13}C) infrared spectroscopy, spectrophotometry (e.g., UV-visible), or mass spectrometry, or by chromatography such as high performance liquid chromatograpy (HPLC) or thin layer chromatography.

[00143] The reactions of the processes described herein can be carried out in suitable solvents which can be readily selected by one of skill in the art of organic synthesis. Suitable solvents can be substantially nonreactive with the starting materials (reactants), the intermediates, or products at the temperatures at which the reactions are carried out, e.g., temperatures which can range from the solvent's freezing temperature to the solvent's boiling temperature. A given reaction can be carried out in one solvent or a mixture of more than one solvent. Depending on the particular reaction step, suitable solvents for a particular reaction step can be selected. In some embodiments, reactions can be carried out in the

absence of solvent, such as when at least one of the reagents is a liquid or gas. Some example solvents suitable for the processes described herein include halogenated hydrocarbons (e.g., methylene chloride), aromatic hydrocarbons (e.g., benzene, toluene, etc.), and ethers (e.g., diethyl ether, tetrahydrofuran).

[00144] The reactions of the processes described herein can be carried out at appropriate temperatures which can be readily determined by the skilled artisan. Reaction temperatures will depend on, for example, the melting and boiling points of the reagents and solvent, if present; the thermodynamics of the reaction (e.g., vigorously exothermic reactions are typically carried out at reduced temperatures); and the kinetics of the reaction (e.g., a high activation energy barrier typically necessitates elevated temperatures). "Elevated temperature" refers to temperatures above room temperature (about 20°C) and "reduced temperature" refers to temperatures below room temperature.

[00145] The reactions of the processes described herein can be carried out in air or under an inert atmosphere. Typically, reactions containing reagents or products that are substantially reactive with air can be carried out using air-sensitive synthetic techniques that are well known to the skilled artisan.

[00146] Upon carrying out preparation of compounds according to the processes described herein, the usual isolation and purification operations such as concentration, filtration, extraction, solid-phase extraction, recrystallization, chromatography, and the like may be used, to isolate the desired products.

Assay and model systems

[00147] *In vitro* or *in vivo* models of infectious diseases are well known by those of skill in the art. For example, for tuberculosis, a rabbit TB model or an *in vitro* Macrophage Model may be used to test for anti-infectious disease activity. Abe et al., (Journal of Immunology, 2003, 171: 1133–1139) discuss other assays suitable for testing compounds for activity against infectious diseases. In some embodiments, an *in vitro* assay can be used as an initial screen, and then inhibitors that are active *in vitro* can be subsequently tested in relevant animal models to assess anti-tuberculosis activity.

[00148] Assays for measuring oxidation of NADH, growth inhibition of *M. tuberculosis* or *M. smegmatis*, inhibition of respiration of *M. tuberculosis* or *M. smegmatis*, or inhibition of replication of *M. tuberculosis* or *M. smegmatis* are described herein. Other methods for achieving similar information are well-known to those of skill in the art.

[00149] Methods for monitoring growth of *M. tuberculosis* or *M. smegmatis* include the use of the BACTEC system to monitor oxygen consumption with fluorescence quenching. Other methods to monitor growth of *M. tuberculosis* or *M. smegmatis* include the BACTEC method using radioactive carbon source as a nutrient and monitoring radioactive carbon dioxide output; plating TB on growth medium and monitoring growth; spectrometer reading optical density (OD=600) of bacilli in liquid medium; culturing of *M. tuberculosis* or *M. smegmatis* with radioactive nulceotide in growth medium (such as radioactive uracil) and monitoring radioactivity of the of *M. tuberculosis* or *M. smegmatis*, among others.

[00150] In order to measure disruption of the electron transport chain, one method is to measuring growth of a variable culture versus the growth of a control and/or measuring loss of viability of a culture over time.

[00151] Models and assays for measuring the effect of inhibitors on the dopamine receptor system are well known to the art-skilled. Exemplary models are discussed, *inter alia*, Hoare *et al*, (Mol Pharmacol. 1996 Nov;50(5):1295-308); Morelli *et al.*, (J Pharmacol Exp Ther. 1992 Jan;260(1):402-8); and Izenwasser *et* al., (J Neurochem. 1995 Apr;64(4):1614-21), among others.

Vaccines

[00152] In some embodiments the present invention also provides recombinant vaccines and immunogenic compositions for the treatment and/or prevention of tuberculosis. In some embodiments, a recombinant vaccine comprises a nucleotide sequence that encodes a *M. tuberculosis* immunogen operably linked to one or more regulatory elements. In some embodiments the immunogen comprises a polypeptide of the *M. tuberculosis* electron transport chain, or a fragment thereof.

[00153] In some embodiments the recombinant vaccine encodes an immunogen comprising a functional fragment of a polypeptide of the *M. tuberculosis* electron transport chain or a non-functional fragment thereof. In some embodiments the nucleotide sequence encodes for a polypeptide comprising a sequence of SEQ ID NOs: 1, 3, 5, 7, 9 or 11. In some embodiments the recombinant vaccine comprises a nucleotide sequence comprising a sequence of SEQ ID NOs: 2, 4, 6, 8, 10 or 12.

[00154] In some embodiments, the recombinant vaccine is a recombinant vaccinia vaccine.

[00155] The present invention also provides methods for inducing an immune response in an animal (*e.g.* human, mouse, dog, cat, monkey, or horse) against a pathogen (*e.g. M. tuberculosis*) comprising administering to the animal a recombinant vaccine.

[00156] Inducing an immune response in a patient can also be used to induce a passive immune response and the antibodies that are generated can be collected from the animal or a patient. The antibodies can be used, *inter alia,* as research and diagnostic tools.

[00157] The present invention also provides methods of inducing an immune response in an animal comprising administering an immunogenic composition comprising an attenuated *M. tuberculosis*.

[00158] In some embodiments the attenuated bacteria comprises a mutated gene of the *M. tuberculosis* electron transport chain. In some embodiments the mutated *M. tuberculosis* electron transport chain gene encodes for a fragment. In some embodiments the mutation (*e.g.* insertion, deletion, frameshift, and the like) can result in the gene having reduced expression as compared to the non-attenuated bacteria. In some embodiments, the protein encoded for by the mutated *M. tuberculosis* electron transport chain gene has diminished activity. In some embodiments, the hydrolytic activity is diminished. In some embodiments, the synthetic activity is diminished. In some embodiments the hydrolytic activity and the synthetic activity is diminished. In some embodiments the mutated *M. tuberculosis* electron transport chain gene encodes for a polypeptide having the sequence of SEQ ID NOs: 1, 3, 5, 7, 9 or 11. In some embodiments, the mutated *M. tuberculosis* electron transport chain gene encodes for a polypeptide that has stimulated activity (*e.g* hydrolysis and/or synthesis) as compared to the polypeptide encoded by the non-attenuated (*e.g.* wild-type) gene.

[00159] In some embodiments the present invention provides recombinant vaccines and attenuated bacteria comprising a knockout of the menaquinone synthesis gene (menABCDEG).

[00160] The present invention also provides methods of inducing an immune response in an animal comprising administering an immunogenic composition comprising a polypeptide, wherein the polypeptide comprises a polypeptide of the *M. tuberculosis* electron transport chain, or fragments thereof. In some embodiments, the fragments are functional. In some embodiments the composition comprises more than one polypeptide. In some embodiments the fragments comprise at least a portion of SEQ ID NOs: 1, 3, 5, 7, 9 or 11. In some embodiments, the polypeptides have diminished and/or stimulated electron transport activity.

[00161] The immunogenic compositions of the present invention can be administered by methods well known to those skilled in the art. In some embodiments, the immunogenic compositions are administered more than once. In some embodiments the immunogenic composition is administered more than once over the span of one day, one week, one month, two months, three months, six months, or one year.

[00162] The present invention also provides methods of protecting an animal from mycobacterial infection comprising administering to the animal an amount of a composition comprising a *M. tuberculosis* electron transport chain modulator effective to protect the animal from the infection.

[00163] The present invention further provides methods of modulating the growth of a pathogen (*e.g.* mycobacteria) comprising administering to the pathogen an amount of a composition comprising a modulator effective to inhibit the growth of the pathogen. In some embodiments the modulator is a *M. tuberculosis* electron transport chain gene or protein modulator.

Polynucleotides

[00164] The present invention provides purified and isolated polynucleotides (*e.g.*, DNA sequences and RNA transcripts, both sense and complementary antisense strands, both single- and double-stranded, including splice variants thereof).

[00165] The invention provides purified and isolated polynucleotides (*e.g.*, cDNA, genomic DNA, synthetic DNA, RNA, or combinations thereof, whether single- or double-stranded) that comprise a nucleotide sequence encoding the amino acid sequence of the polypeptides of the invention. Such polynucleotides are useful for recombinantly expressing the protein and also for detecting expression of the protein in cells (*e.g.*, using Northern hybridization and *in situ* hybridization assays). Such polynucleotides also are useful in the design of antisense and other molecules for the modulation of the gene expression in a cultured cell, a tissue, or an animal; for therapeutic purposes; or to provide a model for diseases or conditions characterized by expression of a particular gene. Specifically excluded from the definition of polynucleotides of the invention are entire isolated, non-recombinant native chromosomes of host cells.

[00166] In some embodiments the polynucleotide has a sequence of SEQ ID NOs: 2, 4, 6, 8, 10 or 12, which correspond to naturally occurring *M. tuberculosis* electron transport chain sequences. It will be appreciated that although the present invention specifically discloses

certain sequences, other polynucleotide sequences exist that also encode other *M. tuberculosis* electron transport chain polypeptides. In some instances, other polynucleotide sequences exist due to the well-known degeneracy of the universal genetic code.

[00167] The invention also provides purified and isolated polynucleotides comprising a nucleotide sequence that encodes a polypeptide, wherein the polynucleotide hybridizes to a polynucleotide having the sequence set forth in sequences of SEQ ID NOs: 2, 4, 6, 8, 10 or 12, or the non-coding strand complementary thereto, under the following hybridization conditions:

(a) hybridization for 16 hours at 42°C in a hybridization solution comprising 50% formamide, 1% SDS, 1 M NaCl, 10% dextran sulfate; and

(b) washing 2 times for 30 minutes each at 60°C in a wash solution comprising 0.1% SSC, 1% SDS. Polynucleotides that encode a human allelic variant are highly preferred.

[00168] The present invention relates to molecules which comprise the gene sequences that encode polypeptides of the *M. tuberculosis* electron transport chain; constructs and recombinant host cells incorporating the gene sequences; the novel *M. tuberculosis* electron transport chain polypeptides encoded by the gene sequences; antibodies to the polypeptides and homologs; kits employing the polynucleotides and polypeptides, and methods of making and using all of the foregoing. In addition, the present invention relates to homologs of the gene sequences and of the polypeptides and methods of making and using the same.

[00169] The invention also comprehends cDNA that is obtained through reverse transcription of an RNA polynucleotide encoding *M. tuberculosis* electron transport chain polypeptides (conventionally followed by second strand synthesis of a complementary strand to provide a double-stranded DNA).

[00170] In some embodiments DNA sequences encoding *M. tuberculosis* electron transport chain polypeptides are selected from SEQ ID NOs: 1, 3, 5, 7, 9 or 11. In some embodiments DNA of the invention comprises a double stranded molecule along with the complementary molecule (the "non-coding strand" or "complement") having a sequence unambiguously deducible from the coding strand according to Watson-Crick base-pairing rules for DNA.

[00171] In some embodiments, the isolated nucleic acid comprises a nucleotide sequence of SEQ ID NOS: 2, 4, 6, 8, 10 or 12, and fragments thereof, that encode a polypeptide having a sequence of SEQ ID NOS: 1, 3, 5, 7, 9 or 11, or fragments thereof.

[00172] The invention further embraces species homologs of the *M. tuberculosis* electron transport chain DNA. Species homologs, sometimes referred to as "orthologs," in general, share at least 35%, at least 40%, at least 45%, at least 50%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% homology with *M. tuberculosis* electron transport chain DNA of the invention. Generally, percent sequence "homology" with respect to polynucleotides of the invention may be calculated as the percentage of nucleotide bases in the candidate sequence that are identical to nucleotides in the *M. tuberculosis* electron transport chain sequences set forth in sequences of SEQ ID NOs: 2, 4, 6, 8, 10 or 12, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity.

[00173] The disclosure herein of full-length polynucleotides encoding *M. tuberculosis* electron transport chain polypeptides makes readily available to the worker of ordinary skill in the art other fragments of the full-length polynucleotide.

[00174] In some embodiments the present invention provides an isolated nucleic acid molecule comprising a sequence homologous to sequences of SEQ ID NOs: 2, 4, 6, 8, 10 or 12, and fragments thereof. In some embodiments provides an isolated nucleic acid molecule comprising a sequence of SEQ ID NOs: 2, 4, 6, 8, 10 or 12, and fragments thereof.

[00175] As used in the present invention, fragments of *M. tuberculosis* electron transport chain-encoding polynucleotides comprise at least 10, and preferably at least 12, 14, 16, 18, 20, 25, 50, 75, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, or 2000 consecutive nucleotides of a polynucleotide encoding a *M. tuberculosis* electron transport chain polypeptide. In some embodiments, fragment polynucleotides of the invention comprise sequences unique to the *M. tuberculosis* electron transport chain polypeptide-encoding polynucleotide sequence, and therefore hybridize under highly stringent or moderately stringent conditions only (*i.e.*, "specifically") to polynucleotides encoding *M. tuberculosis* electron transport chain polypeptides (or fragments thereof). Sequences unique to polynucleotides of the invention are recognizable through sequence comparison to other known polynucleotides, and can be identified through use of alignment programs routinely utilized in the art, *e.g.*, those made available in public sequence databases. Such sequences also are recognizable from Southern hybridization analyses to determine the number of fragments of genomic DNA to which a polynucleotide will

hybridize. Polynucleotides of the invention can be labeled in a manner that permits their detection, including radioactive, fluorescent, and enzymatic labeling.

[00176] Fragment polynucleotides are particularly useful as probes for detection of full-length or fragments of *M. tuberculosis* electron transport chain polynucleotides. One or more polynucleotides can be included in kits that are used to detect the presence of a polynucleotide encoding *M. tuberculosis* electron transport chain polypeptides, or used to detect variations in a polynucleotide sequence encoding *M. tuberculosis* electron transport chain polypeptides.

[00177] The invention also embraces DNAs encoding *M. tuberculosis* electron transport chain polypeptides that hybridize under moderately stringent or high stringency conditions to the non-coding strand, or complement, of the polynucleotides set forth in sequences of SEQ ID NOs: 2, 4, 6, 8, 10 or 12.

[00178] In some embodiments, highly stringent hybridization conditions are as follows: hybridization at 42°C in a hybridization solution comprising 50% formamide, 1% SDS, 1 M NaCl, 10% Dextran sulfate, and washing twice for 30 minutes at 60°C in a wash solution comprising 0.1X SSC and 1% SDS. It is understood in the art that conditions of equivalent stringency can be achieved through variation of temperature and buffer, or salt concentration as described Ausubel *et al.* (Eds.), Protocols in Molecular Biology, John Wiley & Sons (1994), pp. 6.0.3 to 6.4.10. Modifications in hybridization conditions can be empirically determined or precisely calculated based on the length and the percentage of guanosine/cytosine (GC) base pairing of the probe. The hybridization conditions can be calculated as described in Sambrook, *et al.*, (Eds.), Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York (1989), pp. 9.47 to 9.51.

[00179] The polynucleotide sequence information provided by the invention makes possible large-scale expression of the encoded polypeptide by techniques well known and routinely practiced in the art.

Vectors

[00180] Another aspect of the present invention is directed to vectors, or recombinant expression vectors, comprising any of the nucleic acid molecules described above. Vectors are used herein either to amplify DNA or RNA encoding *M. tuberculosis* electron transport chain polypeptides and/or to express DNA which encodes *M. tuberculosis* electron transport

chain polypeptides. In some embodiments, vectors include, but are not limited to, plasmids, phages, cosmids, episomes, viral particles or viruses, and integratable DNA fragments (*i.e.*, fragments integratable into the host genome by homologous recombination). In some embodiments viral particles include, but are not limited to, adenoviruses, baculoviruses, parvoviruses, herpesviruses, poxviruses, adeno-associated viruses, Semliki Forest viruses, vaccinia viruses, and retroviruses. Preferred expression vectors include, but are not limited to, pcDNA3 (Invitrogen). Other expression vectors include, but are not limited to, pSPORT™ vectors, pGEM™ vectors (Promega), pPROEXvectors™ (LTI, Bethesda, MD), Bluescript™ vectors (Stratagene), pQE™ vectors (Qiagen), pSE420™ (Invitrogen), and pYES2™(Invitrogen).

[00181] Expression constructs preferably comprise *M. tuberculosis* electron transport chain-encoding polynucleotides operatively linked to an endogenous or exogenous expression control DNA sequence and a transcription terminator. Expression control DNA sequences include promoters, enhancers, operators, and regulatory element binding sites generally, and are typically selected based on the expression systems in which the expression construct is to be utilized. Preferred promoter and enhancer sequences are generally selected for the ability to increase gene expression, while operator sequences are generally selected for the ability to regulate gene expression. Expression constructs of the invention may also include sequences encoding one or more selectable markers that permit identification of host cells bearing the construct. Expression constructs may also include sequences that facilitate, and preferably promote, homologous recombination in a host cell. Preferred constructs of the invention also include sequences necessary for replication in a host cell.

[00182] Expression constructs are utilized for production of an encoded protein, but may also be utilized simply to amplify a *M. tuberculosis* electron transport chain-encoding polynucleotide sequence. In some embodiments, the vector is an expression vector wherein the polynucleotide of the invention is operatively linked to a polynucleotide comprising an expression control sequence. Autonomously replicating recombinant expression constructs such as plasmid and viral DNA vectors incorporating polynucleotides of the invention are also provided. In some embodiments expression vectors are replicable DNA constructs in which a DNA sequence encoding a *M. tuberculosis* electron transport chain polypeptide is operably linked or connected to suitable control sequences capable of effecting the expression of the *M. tuberculosis* electron transport chain polypeptide in a suitable host. DNA regions

are operably linked or connected when they are functionally related to each other. For example, a promoter is operably linked or connected to a coding sequence if it controls the transcription of the sequence. Amplification vectors do not require expression control domains, but rather need only the ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants. The need for control sequences in the expression vector will vary depending upon the host selected and the transformation method chosen. Generally, control sequences include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding and sequences which control the termination of transcription and translation.

[00183] In some embodiments, vectors contain a promoter that is recognized by the host organism. The promoter sequences of the present invention may be prokaryotic, eukaryotic or viral. Examples of suitable prokaryotic sequences include the P_R and P_L promoters of bacteriophage lambda (The bacteriophage Lambda, Hershey, A. D., Ed., Cold Spring Harbor Press, Cold Spring Harbor, NY (1973), which is incorporated herein by reference in its entirety; Lambda II, Hendrix, R. W., Ed., Cold Spring Harbor Press, Cold Spring Harbor, NY (1980), which is incorporated herein by reference in its entirety); the trp, recA, heat shock, and lacZ promoters of *E. coli* and the SV40 early promoter (Benoist *et al. Nature*, 1981, *290*, 304-310, which is incorporated herein by reference in its entirety). Additional promoters include, but are not limited to, mouse mammary tumor virus, long terminal repeat of human immunodeficiency virus, maloney virus, cytomegalovirus immediate early promoter, Epstein Barr virus, Rous sarcoma virus, human actin, human myosin, human hemoglobin, human muscle creatine, and human metalothionein.

[00184] Additional regulatory sequences can also be included in vectors. In some embodiments, examples of suitable regulatory sequences are represented by the Shine-Dalgarno of the replicase gene of the phage MS-2 and of the gene cII of bacteriophage lambda. The Shine-Dalgarno sequence may be directly followed by DNA encoding *M. tuberculosis* electron transport chain polypeptides or fragments thereof and result in the expression of the mature *M. tuberculosis* electron transport chain polypeptide or fragments thereof.

[00185] Moreover, suitable expression vectors can include an appropriate marker that allows the screening of the transformed host cells. The transformation of the selected host is

carried out using any one of the various techniques well known to the expert in the art and described in Sambrook *et al.*, *supra*.

[00186] An origin of replication can also be provided either by construction of the vector to include an exogenous origin or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter may be sufficient. Alternatively, rather than using vectors which contain viral origins of replication, one skilled in the art can transform mammalian cells by the method of co-transformation with a selectable marker and *M. tuberculosis* electron transport chain DNA. An example of a suitable marker is dihydrofolate reductase (DHFR) or thymidine kinase (*see*, U.S. Patent No. 4,399,216).

[00187] Nucleotide sequences encoding *M. tuberculosis* electron transport chain polypeptides may be recombined with vector DNA in accordance with conventional techniques, including blunt-ended or staggered-ended termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesiderable joining, and ligation with appropriate ligases. Techniques for such manipulation are disclosed by Sambrook et al., *supra* and are well known in the art. Methods for construction of mammalian expression vectors are disclosed in, for example, Okayama *et al.*, *Mol. Cell. Biol.*, 1983, *3*, 280, Cosman *et al.*, *Mol. Immunol.*, 1986, *23*, 935, Cosman *et al.*, *Nature*, 1984, *312*, 768, EP-A-0367566, and WO 91/18982, each of which is incorporated herein by reference in its entirety.

Host cells

[00188] According to another aspect of the invention, host cells are provided, including prokaryotic and eukaryotic cells, comprising a polynucleotide of the invention (or vector of the invention) in a manner that permits expression of the encoded *M. tuberculosis* electron transport chain polypeptide or fragments thereof. Polynucleotides of the invention may be introduced into the host cell as part of a circular plasmid, or as linear DNA comprising an isolated protein coding region or a viral vector. Methods for introducing DNA into the host cell that are well known and routinely practiced in the art include transformation, transfection, electroporation, nuclear injection, or fusion with carriers such as liposomes, micelles, ghost cells, and protoplasts. Expression systems of the invention include bacterial, yeast, fungal, plant, insect, invertebrate, vertebrate, and mammalian cells systems. In some embodiments the bacterial system is *M. smegmatis* or *M. tuberculosis*.

[00189] The invention provides host cells that are transformed or transfected (stably or transiently) with polynucleotides of the invention or vectors of the invention. As stated above, such host cells are useful for amplifying the polynucleotides and also for expressing the *M. tuberculosis* electron transport chain polypeptide or fragments thereof encoded by the polynucleotide.

[00190] One of skill in the art can introduce nucleic acid molecules into mycobacteria (e.g. *M. tuberculosis* or *M. smegmatis*) by methods known to those of skill in the art (see, for example, Proc Natl Acad Sci U S A. 1991 Apr 15; 88(8):3111-5; Mol Microbiol. 1989 Jan;3(1):29-34, and Nutrition. 1995 Sep-Oct;11(5 Suppl):670-3). These methods can be used, *inter alia*, for the expression of exogenous proteins and for homologous recombination.

[00191] In still another related embodiment, the invention provides a method for producing a *M. tuberculosis* electron transport chain polypeptide (or fragment thereof) comprising the steps of growing a host cell of the invention in a nutrient medium and isolating the polypeptide or variant thereof from the cell or the medium.

[00192] According to some aspects of the present invention, transformed host cells having an expression vector comprising any of the nucleic acid molecules described above are provided. Expression of the nucleotide sequence occurs when the expression vector is introduced into an appropriate host cell. Suitable host cells for expression of the polypeptides of the invention include, but are not limited to, prokaryotes, yeast, and eukaryotes. If a prokaryotic expression vector is employed, then the appropriate host cell would be any prokaryotic cell capable of expressing the cloned sequences. Suitable prokaryotic cells include, but are not limited to, bacteria of the genera *Escherichia*, *Bacillus*, *Salmonella*, *Pseudomonas*, *Streptomyces*, *M. smegmatis* and *M. tuberculosis*.

[00193] If an eukaryotic expression vector is employed, then the appropriate host cell would be any eukaryotic cell capable of expressing the cloned sequence. In some embodiments, eukaryotic cells are cells of higher eukaryotes. Suitable eukaryotic cells include, but are not limited to, non-human mammalian tissue culture cells and human tissue culture cells. In some embodiments, host cells include, but are not limited to, insect cells, HeLa cells, Chinese hamster ovary cells (CHO cells), African green monkey kidney cells (COS cells), human HEK-293 cells, and murine 3T3 fibroblasts. Propagation of such cells in cell culture has become a routine procedure (*see*, Tissue Culture, Academic Press, Kruse and Patterson, eds. (1973), which is incorporated herein by reference in its entirety).

[00194] In addition, a yeast host may be employed as a host cell. Preferred yeast cells include, but are not limited to, the genera *Saccharomyces*, *Pichia*, and *Kluveromyces*. Preferred yeast hosts are *S. cerevisiae* and *P. pastoris*. Preferred yeast vectors can contain an origin of replication sequence from a 2T yeast plasmid, an autonomously replication sequence (ARS), a promoter region, sequences for polyadenylation, sequences for transcription termination, and a selectable marker gene. Shuttle vectors for replication in both yeast and *E. coli* are also included herein.

[00195] Alternatively, insect cells may be used as host cells. In some embodiments, the polypeptides of the invention are expressed using a baculovirus expression system (*see*, Luckow *et al.*, *Bio/Technology*, 1988, *6*, 47, Baculovirus Expression Vectors: A Laboratory Manual, O'Rielly *et al.* (Eds.), W.H. Freeman and Company, New York, 1992, and U.S. Patent No. 4,879,236, each of which is incorporated herein by reference in its entirety). In addition, the MAXBAC™ complete baculovirus expression system (Invitrogen) can, for example, be used for production in insect cells.

[00196] Host cells of the invention are a valuable source of immunogen for development of antibodies specifically immunoreactive with *M. tuberculosis* electron transport chain polypeptides. Host cells of the invention are also useful in methods for the large-scale production of *M. tuberculosis* electron transport chain polypeptides wherein the cells are grown in a suitable culture medium and the desired polypeptide products are isolated from the cells, or from the medium in which the cells are grown, by purification methods known in the art, *e.g.*, conventional chromatographic methods including immunoaffinity chromatography, protein affinity chromatography, hydrophobic interaction chromatography, lectin affinity chromatography, size exclusion filtration, cation or anion exchange chromatography, high pressure liquid chromatography (HPLC), reverse phase HPLC, and the like. Still other methods of purification include those methods wherein the desired protein is expressed and purified as a fusion protein having a specific tag, label, or chelating moiety that is recognized by a specific binding partner or agent. The purified protein can be cleaved to yield the desired protein, or can be left as an intact fusion protein. Cleavage of the fusion component may produce a form of the desired protein having additional amino acid residues as a result of the cleavage process.

[00197] Knowledge of *M. tuberculosis* electron transport chain DNA sequences allows for modification of cells to permit, or increase, expression of endogenous *M. tuberculosis*

electron transport chain polypeptides. Cells can be modified (*e.g.*, by homologous recombination) to provide increased expression by replacing, in whole or in part, the naturally occurring *M. tuberculosis* electron transport chain polynucleotide's promoter with all or part of a heterologous promoter so that the cells express *M. tuberculosis* electron transport chain polypeptides at higher levels. The heterologous promoter is inserted in such a manner that it is operatively linked to endogenous *M. tuberculosis* electron transport chain-encoding sequences. (See, for example, PCT International Publication No. WO 94/12650, PCT International Publication No. WO 92/20808, and PCT International Publication No. WO 91/09955.) It is also contemplated that, in addition to heterologous promoter DNA, amplifiable marker DNA (*e.g.*, *ada*, *dhfr* and the multifunctional CAD gene which encodes carbamoyl phosphate synthase, aspartate transcarbamylase, and dihydroorotase) and/or intron DNA may be inserted along with the heterologous promoter DNA. If linked to the *M. tuberculosis* electron transport chain coding sequence, amplification of the marker DNA by standard selection methods results in co-amplification of the *M. tuberculosis* electron transport chain coding sequences in the cells.

Knock-outs

[00198] The DNA sequence information provided by the present invention also makes possible the development (*e.g.*, by homologous recombination or "knock-out" strategies; see Capecchi, *Science 244*:1288-1292 (1989), which is incorporated herein by reference) of bacteria that fail to express functional *M. tuberculosis* electron transport chain polypeptides or that express a variant of a *M. tuberculosis* electron transport chain polypeptide. Such models are useful for studying the *in vivo* activities of *M. tuberculosis* electron transport chain polypeptides and modulators of *M. tuberculosis* electron transport chain polypeptide activity.

Antisense Inhibitors

[00199] In some embodiments the inhibitor is an antisense oligonucleotide comprising at least 80% sequence homology to the complement of a nucleic acid molecule encoding a *M. tuberculosis* electron transport chain polypeptide (SEQ ID NO:2, 4, 6, 8, 10 or 12), wherein the antisense oligonucleotide specifically hybridizes to the nucleic acid molecule and inhibits *M. tuberculosis* electron transport chain polypeptide mRNA levels by at least 50% in *M. tuberculosis*. In some embodiments the antisense oligonucleotide specifically hybridizes with the 5' UTR, start codon region, intron/exon region, coding region, stop codon region, or

3'UTR of said polynucleotide. In some embodiments the antisense oligonucleotide comprises at least 95% sequence homology to the complement of a nucleic acid molecule encoding *M. tuberculosis* electron transport chain polypeptide (SEQ ID NO:2, 4, 6, 8, 10 or 12). In some embodiments the antisense oligonucleotide specifically hybridizes to an electron transport chain polynucleotide, wherein said electron transport chain polynucleotide is type II NADH dehydrogenase, menaquinone, flavin adenine dinucleotide, bc1 complex, cytochrome bd oxidase, fumarate reductase, or nitrate reductase. In some embodiments the antisense oligonucleotide inhibits *M. tuberculosis* electron transport chain polypeptide mRNA levels by at least 75% or at least 90% in *M. tuberculosis*.

[00200] Also made available by the invention are antisense polynucleotides that recognize and hybridize to polynucleotides encoding *M. tuberculosis* electron transport chain polypeptides. Full-length and fragment anti-sense polynucleotides are provided. Fragment antisense molecules of the invention include (i) those that specifically recognize and hybridize to *M. tuberculosis* electron transport chain RNA (as determined by sequence comparison of DNA encoding *M. tuberculosis* electron transport chain to DNA encoding other known molecules). Identification of sequences unique to *M. tuberculosis* electron transport chain encoding polynucleotides can be deduced through use of any publicly available sequence database, and/or through use of commercially available sequence comparison programs. After identification of the desired sequences, isolation through restriction digestion or amplification using any of the various polymerase chain reaction techniques well known in the art can be performed. Anti-sense polynucleotides are particularly relevant to regulating expression of *M. tuberculosis* electron transport chain polypeptides by those cells expressing *M. tuberculosis* electron transport chain mRNA.

[00201] Antisense nucleic acids (*e.g.*10 to 30 base-pair oligonucleotides) capable of specifically binding to *M. tuberculosis* electron transport chain polypeptide expression control sequences or *M. tuberculosis* electron transport chain RNA are introduced into cells (*e.g.*, by a viral vector or colloidal dispersion system such as a liposome). The antisense nucleic acid binds to the *M. tuberculosis* electron transport chain target nucleotide sequence in the cell and prevents transcription and/or translation of the target sequence. Phosphorothioate and methylphosphonate antisense oligonucleotides are contemplated for therapeutic use by the invention. The antisense oligonucleotides may be further modified by adding poly-L-lysine, transferrin polylysine, or cholesterol moieties at their 5' end.

Suppression of *M. tuberculosis* electron transport chain polypeptides expression at either the transcriptional or translational level is useful to generate cellular or animal models for diseases/conditions characterized by *M. tuberculosis* electron transport chain polypeptides expression.

[00202] Antisense oligonucleotides, or fragments of sequences of SEQ ID NOs: 2, 4, 6, 8, 10 or 12, or sequences complementary or homologous thereto, derived from the nucleotide sequences of the present invention encoding *M. tuberculosis* electron transport chain polypeptides are useful as diagnostic tools for probing gene expression in various tissues. For example, tissue can be probed *in situ* with oligonucleotide probes carrying detectable groups by conventional autoradiography techniques to investigate native expression of this protein or pathological conditions relating thereto. Antisense oligonucleotides can be directed to domains or regulatory regions of sequences of SEQ ID NOs: 2, 4, 6, 8, 10 or 12, or mRNA corresponding thereto, including, but not limited to, the initiation codon, TATA box, enhancer sequences, and the like.

Polypeptides

[00203] In some embodiments the invention provides purified and isolated *M. tuberculosis* electron transport chain polypeptides encoded by a polynucleotide of the invention. In some embodiments the *M. tuberculosis* electron transport chain polypeptide comprises the amino acid sequence set out in sequences of SEQ ID NOs: 1, 3, 5, 7, 9 or 11, or fragments thereof comprising an epitope specific to the polypeptide. By "epitope specific to" is meant a portion of the *M. tuberculosis* electron transport chain polypeptide that is recognizable by an antibody that is specific for the *M. tuberculosis* electron transport chain polypeptide, as defined in detail below.

[00204] In some embodiments the isolated polypeptide comprises a fragment of an electron transport chain polypeptide, wherein the fragment is at least 10 amino acid residues and comprises at least one epitope of the electron transport chain polypeptide. In some embodiments the electron transport chain polypeptide is a *M. tuberculosis* or *M. smegmatis* electron transport chain polypeptide. In some embodiments the invention provides isolated epitope-bearing fragments of the polypeptide comprising a sequence of SEQ ID NO: 1, 3, 5, 7, 9 or 11, wherein the fragment has at least 10 amino acid residues.

[00205] Although the sequences provided are *M. tuberculosis* sequences, the invention is intended to include within its scope other bacterial forms of *M. tuberculosis* electron transport chain polypeptides.

[00206] The invention also embraces polypeptides that have at least 99%, at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 65%, at least 60%, at least 55% or at least 50% identity and/or homology to the preferred polypeptides of the invention. Percent amino acid sequence "identity" with respect to the preferred polypeptide of the invention is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the residues in the *M. tuberculosis* electron transport chain polypeptide sequence after aligning both sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Percent sequence "homology" with respect to the preferred polypeptide of the invention is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the residues in the *M. tuberculosis* electron transport chain polypeptide sequence after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and also considering any conservative substitutions as part of the sequence identity.

[00207] In one aspect, percent homology is calculated as the percentage of amino acid residues in the smaller of two sequences which align with identical amino acid residue in the sequence being compared, when four gaps in a length of 100 amino acids may be introduced to maximize alignment (Dayhoff, in Atlas of Protein Sequence and Structure, Vol. 5, p. 124, National Biochemical Research Foundation, Washington, D.C. (1972), incorporated herein by reference).

[00208] Polypeptides of the invention may be isolated from natural cell sources or may be chemically synthesized, but can be produced by recombinant procedures involving host cells of the invention. Use of mammalian host cells can be used to provide for such post-translational modifications (*e.g.*, glycosylation, truncation, lipidation, and phosphorylation) as may be needed to confer optimal biological activity on recombinant expression products of the invention. Glycosylated and non-glycosylated forms of *M. tuberculosis* electron transport chain polypeptides are embraced by the invention.

[00209] The invention also embraces variant (or analog) *M. tuberculosis* electron transport chain polypeptides. In one example, insertion variants are provided wherein one or

more amino acid residues supplement a *M. tuberculosis* electron transport chain polypeptide's amino acid sequence. Insertions may be located at either or both termini of the protein, or may be positioned within internal regions of the *M. tuberculosis* electron transport chain polypeptide's amino acid sequence. Insertional variants with additional residues at either or both termini can include, for example, fusion proteins and proteins including amino acid tags or labels.

[00210] Insertion variants include *M. tuberculosis* electron transport chain polypeptides wherein one or more amino acid residues are added to a *M. tuberculosis* electron transport chain polypeptide's acid sequence or to a biologically active fragment thereof.

[00211] Variant products of the invention also include mature *M. tuberculosis* electron transport chain products, *i.e.*, *M. tuberculosis* electron transport chain products wherein leader or signal sequences are removed, with additional amino terminal residues. The additional amino terminal residues may be derived from another protein, or may include one or more residues that are not identifiable as being derived from specific proteins. *M. tuberculosis* electron transport chain products with an additional methionine residue at position -1 (Met^{-1}- *M. tuberculosis* electron transport chain polypeptide) are contemplated, as are variants with additional methionine and lysine residues at positions -2 and -1 (Met^{-2}-Lys^{-1}- *M. tuberculosis* electron transport chain polypeptide). Variants of *M. tuberculosis* electron transport chain polypeptides with additional Met, Met-Lys, Lys residues (or one or more basic residues in general) are particularly useful for enhanced recombinant protein production in bacterial host cells.

[00212] The invention also embraces *M. tuberculosis* electron transport chain polypeptide variants having additional amino acid residues that result from use of specific expression systems. For example, use of commercially available vectors that express a desired polypeptide as part of a glutathione-S-transferase (GST) fusion product provides the desired polypeptide having an additional glycine residue at position -1 after cleavage of the GST component from the desired polypeptide. Variants that result from expression in other vector systems are also contemplated.

[00213] Insertional variants also include fusion proteins wherein the amino terminus and/or the carboxy terminus of a *M. tuberculosis* electron transport chain polypeptide is/are fused to another polypeptide.

[00214] In another aspect, the invention provides deletion variants wherein one or more amino acid residues in a *M. tuberculosis* electron transport chain polypeptide are removed. Deletions can be effected at one or both termini of the *M. tuberculosis* electron transport chain polypeptide, or with removal of one or more non-terminal amino acid residues of *M. tuberculosis* electron transport chain polypeptide. Deletion variants, therefore, include all fragments of a *M. tuberculosis* electron transport chain polypeptide.

[00215] The invention also embraces polypeptide fragments of sequences of SEQ ID NOS:1, 3, 5, 7, 9 or 11, wherein the fragments maintain biological (*e.g.*, hydrolysis and/or synthesis activity) and/or immunological properties of a *M. tuberculosis* electron transport chain polypeptide.

[00216] In some embodiments of the invention, an isolated nucleic acid molecule comprises a nucleotide sequence that encodes a polypeptide comprising an amino acid sequence homologous to sequences of SEQ ID NOs:1, 3, 5, 7, 9 or 11, and fragments thereof, wherein the nucleic acid molecule encoding at least a portion of a *M. tuberculosis* electron transport chain polypeptide.

[00217] As used in the present invention, polypeptide fragments comprise at least about 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 200, 225, 250, 300, 350, 400, 450, or 500 consecutive amino acids of sequences of SEQ ID NOS:1, 3, 5, 7, 9 or 11. In some embodiments, polypeptide fragments display antigenic properties unique to, or specific for, a *M. tuberculosis* electron transport chain polypeptide. Fragments of the invention having the desired biological and immunological properties can be prepared by any of the methods well known and routinely practiced in the art. As discussed above, in some embodiments, the fragments have functional activity.

[00218] In still another aspect, the invention provides substitution variants of *M. tuberculosis* electron transport chain polypeptides. Substitution variants include those polypeptides wherein one or more amino acid residues of a *M. tuberculosis* electron transport chain polypeptide are removed and replaced with alternative residues. In one aspect, the substitutions are conservative in nature; however, the invention embraces substitutions that are also non-conservative. Conservative substitutions for this purpose may be defined as set forth below.

[00219] Variant polypeptides include those wherein conservative substitutions have been introduced by modification of polynucleotides encoding polypeptides of the invention.

Amino acids can be classified according to physical properties and contribution to secondary and tertiary protein structure. A conservative substitution is recognized in the art as a substitution of one amino acid for another amino acid that has similar properties. Exemplary conservative substitutions are set out in Table 1 of WO 97/09433, page 10, published March 13, 1997 (PCT/GB96/02197, filed 9/6/96). Alternatively, conservative amino acids can be grouped as described in Lehninger, [Biochemistry, Second Edition; Worth Publishers, Inc. NY, NY (1975), pp.71-77].

[00220] It should be understood that the definition of polypeptides of the invention is intended to include polypeptides bearing modifications other than insertion, deletion, or substitution of amino acid residues. By way of example, the modifications may be covalent in nature, and include for example, chemical bonding with polymers, lipids, other organic, and inorganic moieties. Such derivatives may be prepared to increase circulating half-life of a polypeptide, or may be designed to improve the targeting capacity of the polypeptide for desired cells, tissues, or organs. Similarly, the invention further embraces *M. tuberculosis* electron transport chain polypeptides that have been covalently modified to include one or more water-soluble polymer attachments such as polyethylene glycol, polyoxyethylene glycol, or polypropylene glycol. Variants that display activity properties of native *M. tuberculosis* electron transport chain polypeptides and are expressed at higher levels, as well as variants that provide for constitutively active *M. tuberculosis* electron transport chain polypeptides, can be useful in assays of the invention; the variants are also useful in providing cellular, tissue and animal models of diseases/conditions characterized by aberrant *M. tuberculosis* electron transport chain polypeptide activity.

[00221] In a related embodiment, the present invention provides compositions comprising purified polypeptides of the invention. In some embodiments, compositions comprise, in addition to the polypeptide of the invention, a pharmaceutically acceptable (*i.e.*, sterile and non-toxic) liquid, semisolid, or solid diluent that serves as a pharmaceutical vehicle, excipient, or medium. Any diluent known in the art may be used. Exemplary diluents include, but are not limited to, water, saline solutions, polyoxyethylene sorbitan monolaurate, magnesium stearate, methyl- and propylhydroxybenzoate, talc, alginates, starches, lactose, sucrose, dextrose, sorbitol, mannitol, glycerol, calcium phosphate, mineral oil, and cocoa butter.

[00222] Variants that display activity properties of native *M. tuberculosis* electron transport chain polypeptides and are expressed at higher levels, as well as variants that provide for constitutively active *M. tuberculosis* electron transport chain polypeptides, are particularly useful in assays of the invention; the variants are also useful in assays of the invention and in providing cellular, tissue and animal models of diseases/conditions characterized by aberrant *M. tuberculosis* electron transport chain polypeptide activity.

Antibodies

[00223] In some embodiments the inhibitor is an antibody (*e.g.*, monoclonal and polyclonal antibodies, single chain antibodies, chimeric antibodies, bifunctional/bispecific antibodies, humanized antibodies, human antibodies, and complementary determining region (CDR)-grafted antibodies, including compounds which include CDR sequences which specifically recognize a polypeptide of the invention) specific for *M. tuberculosis* electron transport chain polypeptides or fragments thereof. In some embodiments antibodies of the invention are human antibodies that are produced and identified according to methods described in WO93/11236, published June 20, 1993, which is incorporated herein by reference in its entirety. Antibody fragments, including Fab, Fab′, $F(ab')_2$, and F_v, are also provided by the invention. The term "specific for," when used to describe antibodies of the invention, indicates that the variable regions of the antibodies of the invention recognize and bind *M. tuberculosis* electron transport chain polypeptides exclusively. It will be understood that specific antibodies may also interact with other proteins (for example, *S. aureus* protein A or other antibodies in ELISA techniques) through interactions with sequences outside the variable region of the antibodies, and, in particular, in the constant region of the molecule. Screening assays to determine binding specificity of an antibody of the invention are well known and routinely practiced in the art. For a comprehensive discussion of such assays, see Harlow *et al.* (Eds.), Antibodies A Laboratory Manual; Cold Spring Harbor Laboratory; Cold Spring Harbor, NY (1988), Chapter 6. Antibodies that recognize and bind fragments of the *M. tuberculosis* electron transport chain polypeptides of the invention are also contemplated, provided that the antibodies are specific for *M. tuberculosis* electron transport chain polypeptides. Antibodies of the invention can be produced using any method well known and routinely practiced in the art.

[00224] The present invention also provides antibodies specific for the *M. tuberculosis* electron transport chain polypeptides of the present invention. In some embodiments the

antibody is obtained by immunization of a subject with the epitope-bearing fragment of the invention. In some embodiments the antibody recognizes at least one region of an electron transport chain polypeptide comprising a sequence of SEQ ID NO: 1, 3, 5, 7, 9 or 11. In some embodiments the antibody binds to a catalytic, hydrolytic or binding region of an electron transport chain polypeptide. In some embodiments the antibody is a monoclonal, polyclonal, chimeric, humanized, single-chain antibody or a Fab fragment. In some embodiments the antibody is labeled.

[00225] In some embodiments the binding affinity of said antibody is less than about 1 x 10^5 K_a for a polypeptide other than an electron transport chain polypeptide.

[00226] Antibody specificity is described in greater detail below. However, it should be emphasized that antibodies that can be generated from polypeptides that have previously been described in the literature and that are capable of fortuitously cross-reacting with *M. tuberculosis* electron transport chain polypeptides (*e.g.*, due to the fortuitous existence of a similar epitope in both polypeptides) are considered "cross-reactive" antibodies. Such cross-reactive antibodies are not antibodies that are "specific" for *M. tuberculosis* electron transport chain polypeptides. The determination of whether an antibody is specific for a *M. tuberculosis* electron transport chain polypeptide or is cross-reactive with another known protein is made using any of several assays, such as Western blotting assays, that are well known in the art. In some embodiments, the antibodies are specific for the polypeptides encoded by the nucleic acid molecules wherein the nucleic acid molecule comprises SEQ ID NOs: 2, 4, 6, 8, 10 or 12. In some embodiments, the antibodies are specific for the polypeptides having a sequence of SEQ ID NOs. 1, 3, 5, 7, 9 or 11.

[00227] Antibodies are said to be "specifically binding" if: 1) they exhibit a threshold level of binding activity, and/or 2) they do not significantly cross-react with known related polypeptide molecules. The binding affinity of an antibody can be readily determined by one of ordinary skill in the art, for example, by Scatchard analysis (Scatchard, *Ann. NY Acad. Sci.* 51: 660-672, 1949). In some embodiments, the antibodies of the present invention bind to a M. tuberculosis electron transport chain polypeptide or fragment thereof at least 10^3, more preferably at least 10^4, more preferably at least 10^5, and even more preferably at least 10^6 fold higher than to other proteins.

[00228] In some embodiments, the antibodies of the present invention do not specifically bind to (or recognize) related polypeptides. Related polypeptides may be polypeptides with

similar activity to electron transport chain polypeptides, other polypeptides from mycobacteria, or other polypeptides known to interact with compositions which modulate the electron transport chain (e.g. polypeptides in the dopaminergic system), using a standard Western blot analysis (Ausubel *et al.*). In some embodiments antibodies may be screened against dopamine receptor polypeptides to isolate an antibody population that specifically binds to electron transport chain polypeptides. For example, antibodies specific to electron transport chain polypeptides will flow through a column comprising *M. tuberculosis* polypeptides (with the exception of electron transport chain polypeptides) adhered to insoluble matrix under appropriate buffer conditions. Such screening allows isolation of polyclonal and monoclonal antibodies non-crossreactive to related polypeptides (Antibodies: A Laboratory Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988; Current Protocols in Immunology, Cooligan et al. (eds.), National Institutes of Health, John Wiley and Sons, Inc., 1995). Screening and isolation of specific antibodies is well known in the art (see, Fundamental Immunology, Paul (eds.), Raven Press, 1993; Getzoff et al., Adv. in Immunol. 43: 1-98, 1988; Monoclonal Antibodies: Principles and Practice, Goding, J. W. (eds.), Academic Press Ltd., 1996; Benjamin et al., Ann. Rev. Immunol. 2: 67-101, 1984). Representative examples of such assays include: concurrent immunoelectrophoresis, radioimmunoassay (RIA), radioimmunoprecipitation, enzyme-linked immunosorbent assay (ELISA), dot blot or Western blot assay, inhibition or competition assay, and sandwich assay.

[00229] In some embodiments, the antibodies of the present invention have at least about 1000 fold, and at least about 10,000 fold greater affinity for electron transport chain polypeptides than for related polypeptides. In some embodiments, the binding affinity of an antibody of the present invention is less than about 1×10^5 K_a, less than about 1×10^4 K_a, and preferably less than 1×10^3 K_a, for a related polypeptide other than an electron transport chain polypeptide.

[00230] In some embodiments, the invention provides monoclonal antibodies. Hybridomas that produce such antibodies also are intended as aspects of the invention. In yet another variation, the invention provides a humanized antibody. Humanized antibodies are useful for *in vivo* therapeutic indications.

[00231] In some embodiments, the invention provides a cell-free composition comprising polyclonal antibodies, wherein at least one of the antibodies is an antibody of the invention specific for *M. tuberculosis* electron transport chain polypeptides. Antisera isolated from an

animal is an exemplary composition, as is a composition comprising an antibody fraction of an antisera that has been resuspended in water or in another diluent, excipient, or carrier.

[00232] In still another related embodiment, the invention provides an anti-idiotypic antibody specific for an antibody that is specific for *M. tuberculosis* electron transport chain polypeptides.

[00233] It is well known that antibodies contain relatively small antigen binding domains that can be isolated chemically or by recombinant techniques. Such domains are useful *M. tuberculosis* electron transport chain polypeptide binding molecules themselves, and also may be reintroduced into human antibodies, or fused to toxins or other polypeptides. Thus, in still another embodiment, the invention provides a polypeptide comprising a fragment of a *M. tuberculosis* electron transport chain polypeptide-specific antibody, wherein the fragment and the polypeptide bind to the *M. tuberculosis* electron transport chain polypeptides. By way of non-limiting example, the invention provides polypeptides that are single chain antibodies and CDR-grafted antibodies.

[00234] Non-human antibodies may be humanized by any of the methods known in the art. In one method, the non-human CDRs are inserted into a human antibody or consensus antibody framework sequence. Further changes can then be introduced into the antibody framework to modulate affinity or immunogenicity.

[00235] Antibodies of the present invention may function through different mechanisms. In some embodiments, antibodies trigger antibody-dependent cellular cytotoxicity (ADCC), a lytic attack on antibody-targeted cells. In some embodiments, antibodies have multiple therapeutic functions, including, for example, antigen-binding, induction of apoptosis, and complement-dependent cellular cytotoxicity (CDC).

[00236] Antibodies of the invention are useful for, *e.g.*, therapeutic purposes (by modulating activity of a *M. tuberculosis* electron transport chain polypeptide), diagnostic purposes to detect or quantitate *M. tuberculosis* electron transport chain polypeptides, and purification of *M. tuberculosis* electron transport chain polypeptides. Kits comprising an antibody of the invention for any of the purposes described herein are also comprehended. In general, a kit of the invention also includes a control antigen for which the antibody is immunospecific.

Electron Transport Chain Polypeptide Epitopes

[00237] Linear epitopes of electron transport chain polypeptides for antibody recognition and preparation can be identified by any of numerous methods known in the art. Some example methods include probing antibody-binding ability of peptides derived from the amino acid sequence of the polypeptide. Binding can be assessed by using BIACORE or ELISA methods. Other techniques include exposing peptide libraries on a solid support ("chip") to antibodies and detecting binding through any of multiple methods used in solid-phase screening. Additionally, phage display can be used to screen a library of peptides with selection of epitopes after several rounds of biopanning. Suitable antibody neutralizing agents according to the present invention can recognize linear or conformational epitopes, or combinations thereof.

[0001] Methods of predicting potential epitopes to which an antibody of the invention can bind are well-known to those of skill in the art and include without limitation, Kyte-Doolittle Analysis (Kyte, J. and Dolittle, R.F., J. Mol. Biol. (1982) 157:105-132), Hopp and Woods Analysis (Hopp, T.P. and Woods, K.R., Proc. Natl. Acad. Sci. USA (1981) 78:3824-3828; Hopp, T.J. and Woods, K.R., Mol. Immunol. (1983) 20:483-489.; Hopp, T.J., J. Immunol. Methods (1986) 88:1-18.), Jameson-Wolf Analysis (Jameson, B.A. and Wolf, H., Comput. Appl. Biosci. (1988) 4:181-186.), and Emini Analysis (Emini, E.A., Schlief, W.A., Colonno, R.J. and Wimmer, E., Virology (1985) 140:13-20.).

Compositions

[00238] Expression of *M. tuberculosis* electron transport chain polypeptides is likely connected to the pathogenesis of tuberculosis. It is contemplated that, preventing the expression of, or inhibiting the activity of, *M. tuberculosis* electron transport chain polypeptides will be useful in treating tuberculosis. It is contemplated that antisense therapy or gene therapy could be applied to negatively regulate the expression of *M. tuberculosis* electron transport chain polypeptides.

[00239] Another aspect of the present invention is directed to compositions, including pharmaceutical compositions, comprising any of the nucleic acid molecules or recombinant expression vectors described above and an acceptable carrier or diluent. In some embodiments, the carrier or diluent is pharmaceutically acceptable. Suitable carriers are described in the most recent edition of *Remington's Pharmaceutical Sciences*, A. Osol, a standard reference text in this field, which is incorporated herein by reference in its entirety. In some embodiments, examples of such carriers or diluents include, but are not limited to,

water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Liposomes and nonaqueous vehicles such as fixed oils may also be used. The formulations are sterilized by commonly used techniques.

[00240] Also within the scope of the invention are compositions comprising polypeptides, polynucleotides, or antibodies of the invention that have been formulated with, *e.g.*, a pharmaceutically acceptable carrier.

[00241] The invention also provides methods of using antibodies of the invention. For example, the invention provides a method for modulating the activity of a *M. tuberculosis* electron transport chain polypeptide comprising the step of contacting the *M. tuberculosis* electron transport chain polypeptide with an antibody specific for the *M. tuberculosis* electron transport chain polypeptide, under conditions wherein the antibody binds the protein.

Formulation, Dose and Administration

[00242] Suitable formulations for administration of a composition of the invention to a subject include aqueous and non-aqueous sterile injection solutions which can contain anti-oxidants, buffers, bacteriostats, antibacterial and antifungal agents that render the formulation isotonic with the bodily fluids of the intended patient (e.g., sugars, salts, and polyalcohols), suspending agents and thickening agents. The formulations can be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and can be stored in a frozen or freeze-dried (lyophilized) condition requiring only the addition of sterile liquid carrier immediately prior to use.

[00243] Compositions useful for injection into a patient include sterile aqueous solutions or dispersions, and sterile powder for the preparation of sterile injectable solutions or dispersions. An injectable composition should be fluid to the extent that administration via a syringe is readily performed. Suitable solvents include water, ethanol, polyol (e.g., glycerol, propylene glycol, and liquid polyethylene glycol), and mixtures thereof. Fluidity can be maintained, for example, by the use of a coating such as lecithin and/or by minimization of particle size.

[00244] An inhibitor of the present invention can be administered to a patient intratumorally, peritumorally, systemically, parenterally (e.g., intravenous injection, intra-muscular injection, intra-arterial injection, and infusion techniques), orally, transdermally (topically), intranasally (inhalation), and intramucosally. A delivery method is selected based on considerations such

as the type of the type of carrier or vector, therapeutic efficacy of the composition, location of target area, and the condition to be treated.

[00245] In some embodiments, the inhibitor is administered orally or intravenously to the patient. In some embodiments, the inhibitor is administered systemically. In some embodiments the inhibitor is administered locally to affected regions.

[00246] Liquid carriers are used in preparing liquid dosage forms such as solutions, suspensions, dispersions, emulsions, syrups, elixirs and pressurized compositions. The active ingredient can be dissolved or suspended in a pharmaceutically acceptable liquid such as water, an organic solvent, a mixture of both, or pharmaceutically acceptable oils or fats. The liquid carrier can contain other suitable pharmaceutical additives such as solubilizers, emulsifiers, buffers, preservatives, sweeteners, flavoring agents, suspending agents, thickening agents, colors, viscosity regulators, stabilizers or osmo-regulators. Suitable examples of liquid carriers for oral and parenteral administration include water (partially containing additives as above, e.g. cellulose derivatives, preferably sodium carboxymethyl cellulose solution); alcohols, including monohydric alcohols such as ethanol and polydric alcohols such as glycols and their derivatives; lecithins and oils such as fractionated coconut oil and arachis oil. For parenteral administration, the liquid carrier for pressurized compositions can be a halogenated hydrocarbon or other pharmaceutically acceptable propellant.

[00247] A liquid pharmaceutical composition such as a syrup or elixir may contain, in addition to one or more liquid carriers and the active ingredients, a sweetening agent such as sucrose, preservatives such as methyl and propyl parabens, a pharmaceutically acceptable dye or coloring agent, or a flavoring agent such as cherry or orange flavoring.

[00248] Compositions for inhalation or insufflation include solutions and suspensions in pharmaceutically acceptable, aqueous or organic solvents, or mixtures thereof, and powders. The liquid or solid compositions may contain suitable pharmaceutically acceptable excipients as described supra. In some embodiments, the compositions are administered by the oral or nasal respiratory route for local or systemic effect. Compositions in can be nebulized by use of inert gases. Nebulized solutions may be breathed directly from the nebulizing device or the nebulizing device can be attached to a face mask tent, or intermittent positive pressure breathing machine. Solution, suspension, or powder compositions can be administered orally or nasally from devices which deliver the formulation in an appropriate manner.

[00249] In some embodiments, an "effective amount" refers to the amount of a therapeutic that is effective in an *in vitro* assay in inhibiting *M. tuberculosis* cell growth, inhibiting *M. tuberculosis* cell respiration, inhibiting *M. tuberculosis* cell replication, and the like. In some embodiments, an "effective amount" inhibits *M. tuberculosis* cell growth, *M. tuberculosis* cell respiration, *M. tuberculosis* cell replication, or combinations thereof, by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 2-fold, at least 5-fold, at least 10-fold, or at least 100-fold.

[00250] In some embodiments, compositions are tested *in vitro* or *in vivo* assays in order to determine an "effective amount." For example, in methods disclosed herein for causing cell death, assays suitable include, without limitation, *in vitro* cell viability assays, including the TUNEL assay or other fluorescent based assays such as Cell-Titer Blue (Promega Corp).

[00251] Actual dosage levels of active ingredients in a therapeutic composition of the invention can be varied so as to administer an amount of the composition that is effective to achieve the desired therapeutic response for a particular subject. Administration regimens can also be varied as required to elicit the desired activity. A single injection or multiple injections can be used. The selected dosage level and regimen will depend upon a variety of factors including the activity of the therapeutic composition, formulation, the route of administration, combination with other drugs or treatments, the disease or disorder to be treated, and the physical condition and prior medical history of the subject being treated. Determination and adjustment of an effective amount or dose, as well as evaluation of when and how to make such adjustments, are known to those of ordinary skill in the art of medicine.

[00252] Generally, treatment is initiated with small dosages which can be increased by small increments until the optimum effect under the circumstances is achieved. Generally, a therapeutic dosage of compositions of the present invention may be from about 1 to about 200 mg/kg twice a week to about once every two weeks. For example, the dosage may be about 1 mg/kg once a week as a 2 ml intravenous injection to about 20 mg/kg once every 3 days. The compounds can be administered in one dose, continuously or intermittently throughout the course of treatment. The compositions maybe administered several times each day, once a day, once a week, or once every two weeks.

[00253] For additional guidance regarding formulation, dose and administration regimen, *see* Berkow et al. (1997) The Merck Manual of Medical Information, Home ed. Merck

Research Laboratories, Whitehouse Station, New Jersey; Goodman et al. (1996) Goodman & Gilman's the Pharmacological Basis of Therapeutics, 9th ed. McGraw-Hill Health Professions Division, New York; Ebadi (1998) CRC Desk Reference of Clinical Pharmacology. CRC Press, Boca Raton, Florida; Katzung (2001) Basic & Clinical Pharmacology, 8th ed. Lange Medical Books/McGraw-Hill Medical Pub. Division, New York; Remington et al. (1975) Remington's Pharmaceutical Sciences, 15th ed. Mack Pub. Co., Easton, Pennsylvania; Speight et al. (1997) Avery's Drug Treatment: A Guide to the Properties; Choice, Therapeutic Use and Economic Value of Drugs in Disease Management, 4th ed. Adis International, Auckland / Philadelphia, Pennsylvania.

[00254] The inhibitors of the present invention may further comprise one or more adjuvants which include, but are not limited to: (1) aluminum salts (alum), (2) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides or bacterial cell wall components), such as for example (a) MF59 containing 5% Squalene, 0.5% Tween 80, and 0.5% Span 85 (b) SAF, containing 10% Squalane, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP, and (c) Ribi™ adjuvant system (RAS), (Ribi Immunochem, Hamilton, Mont.) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS); (3) saponin adjuvants, (4) Complete Freunds Adjuvant (CFA) and Incomplete Freunds Adjuvant (IFA); (5) cytokines, such as interleukins (IL-1, IL-2, etc.), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), etc.; (6) detoxified mutants of a bacterial ADP-ribosylating toxin, and (7) other substances that act as immunostimulating agents to enhance the effectiveness of the composition. Muramyl peptides include, without limitation, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acteyl-normuramyl-L-alanyl-D-isogluatme (nor-MDP), N-acetylmuramyl-L-alanyl-D-isogluatminyl-L-alanine-2-(1'-2'-dipahitoyl-sn-glycero-3-huydroxyphosphoryloxy)-ethylamine (MTP-PE), and the like.

Kits

[00255] The present invention is also directed to kits, including pharmaceutical kits. The kits can comprise any of the nucleic acid molecules described above, any of the polypeptides described above, or any antibody which binds to a polypeptide of the invention as described above, as well as a negative control. The kit preferably comprises additional components,

such as, for example, instructions, solid support, reagents helpful for quantification, and the like.

[00256] In another aspect, the invention features methods for detection of a polypeptide in a sample as a diagnostic tool for diseases or disorders, wherein the method comprises the steps of: (a) contacting the sample with a nucleic acid probe which hybridizes under hybridization assay conditions to a nucleic acid target region of a polypeptide having sequences of SEQ ID NO:1, 3, 5, 7, 9, or 11, said probe comprising the nucleic acid sequence encoding the polypeptide, fragments thereof, and the complements of the sequences and fragments; and (b) detecting the presence or amount of the probe:target region hybrid as an indication of the disease. In some embodiments the disease is tuberculosis.

[00257] Kits may be designed to detect either expression of polynucleotides encoding *M. tuberculosis* electron transport chain polypeptides expressed in the lungs or the *M. tuberculosis* electron transport chain polypeptides themselves in order to identify tissue as being from infected tissue. For example, oligonucleotide hybridization kits can be provided which include a container having an oligonucleotide probe specific for the *M. tuberculosis* electron transport chain-specific DNA and optionally, containers with positive and negative controls and/or instructions. Similarly, PCR kits can be provided which include a container having primers specific for the *M. tuberculosis* electron transport chain-specific sequences, DNA and optionally, containers with size markers, positive and negative controls and/or instructions.

[00258] Hybridization conditions should be such that hybridization occurs only with the genes in the presence of other nucleic acid molecules. Under stringent hybridization conditions only highly complementary nucleic acid sequences hybridize. In some embodiments, such conditions prevent hybridization of nucleic acids having 1 or 2 mismatches out of 20 contiguous nucleotides. Such conditions are defined *supra*.

[00259] Alternatively, immunoassay kits can be provided which have containers container having antibodies specific for the *M. tuberculosis* electron transport chain polypeptide and optionally, containers with positive and negative controls and/or instructions.

[00260] Kits may also be provided useful in the identification of *M. tuberculosis* electron transport chain polypeptide binding partners such as natural ligands or modulators (agonists or antagonists). Substances useful for treatment of disorders or diseases preferably show positive results in one or more *in vitro* assays for an activity corresponding to treatment of the

disease or disorder in question. Substances that modulate the activity of the polypeptides preferably include, but are not limited to, antisense oligonucleotides, agonists and antagonists, and inhibitors of protein kinases.

Methods of inducing immune response

[00261] Another aspect of the present invention is directed to methods of inducing an immune response in a mammal against a polypeptide of the invention by administering to the mammal an amount of the polypeptide sufficient to induce an immune response. The amount will be dependent on the animal species, size of the animal, and the like but can be determined by those skilled in the art.

Methods of identifying compounds that bind *M. tuberculosis* electron transport chain polypeptides or fragments thereof

[00262] The present invention also provides assays to identify compounds that bind *M. tuberculosis* electron transport chain polypeptides. One such assay comprises the steps of: (a) contacting a composition comprising a *M. tuberculosis* electron transport chain polypeptide with a compound suspected of binding a *M. tuberculosis* electron transport chain polypeptide; and (b) measuring binding between the compound and the *M. tuberculosis* electron transport chain polypeptide. In some embodiments, the *M. tuberculosis* electron transport chain polypeptide is modified and comprises only the domains that are necessary for synthesis activity or hydrolysis activity. In some embodiments, the composition comprises a cell expressing a *M. tuberculosis* electron transport chain polypeptide. The binding may be measured directly, *e.g.*, by using a labeled compound, or may be measured indirectly by several techniques, including measuring intracellular activity of the *M. tuberculosis* electron transport chain polypeptide induced by the compound (or measuring changes in the level of *M. tuberculosis* electron transport chain polypeptide activity). Following steps (a) and (b), compounds identified as binding *M. tuberculosis* electron transport chain polypeptides may be tested in other assays including, but not limited to, *in vivo* models, to confirm or quantitate binding to *M. tuberculosis* electron transport chain polypeptides.

[00263] Specific binding molecules, including natural ligands and synthetic compounds, can be identified or developed using isolated or recombinant *M. tuberculosis* electron transport chain polypeptides, *M. tuberculosis* electron transport chain polypeptide fragments as described herein, or in some embodiments, cells expressing such products or fragments. Binding partners can be useful for purifying *M. tuberculosis* electron transport chain

polypeptides and detection or quantification of *M. tuberculosis* electron transport chain polypeptides in fluid and tissue samples using known immunological procedures. Binding molecules are also manifestly useful in modulating (*i.e.*, blocking, inhibiting or stimulating) biological activities of *M. tuberculosis* electron transport chain polypeptides.

[00264] The DNA and amino acid sequence information provided by the present invention also makes possible identification of binding partner compounds with which a *M. tuberculosis* electron transport chain polypeptide (or fragment thereof) or polynucleotide will interact. Methods to identify binding partner compounds include solution assays, *in vitro* assays wherein *M. tuberculosis* electron transport chain polypeptides are immobilized, and cell-based assays. Identification of binding partner compounds of *M. tuberculosis* electron transport chain polypeptides provides candidates for therapeutic or prophylactic intervention in pathologies associated with *M. tuberculosis* electron transport chain polypeptide normal and aberrant biological activity.

[00265] The invention includes several assay systems for identifying *M. tuberculosis* electron transport chain polypeptide binding partners. In solution assays, methods of the invention comprise the steps of (a) contacting a *M. tuberculosis* electron transport chain polypeptide with one or more candidate binding partner compounds and (b) identifying the compounds that bind to the *M. tuberculosis* electron transport chain polypeptide. Identification of the compounds that bind the *M. tuberculosis* electron transport chain polypeptide can be achieved by isolating the *M. tuberculosis* electron transport chain polypeptide/binding partner complex, and separating the binding partner compound from the *M. tuberculosis* electron transport chain polypeptide. An additional step of characterizing the physical, biological, and/or biochemical properties of the binding partner compound is also comprehended in another embodiment of the invention, wherein compounds identified as binding *M. tuberculosis* electron transport chain polypeptide may be tested in other assays including, but not limited to, *in vivo* models, to confirm or quantitate binding to *M. tuberculosis* electron transport chain polypeptide. In one aspect, the *M. tuberculosis* electron transport chain polypeptide/binding partner complex is isolated using an antibody immunospecific for either the *M. tuberculosis* electron transport chain polypeptide or the candidate binding partner compound.

[00266] In still other embodiments, either the *M. tuberculosis* electron transport chain polypeptide or the candidate binding partner compound comprises a label or tag that

facilitates its isolation, and methods of the invention to identify binding partner compounds include a step of isolating the *M. tuberculosis* electron transport chain polypeptide/binding partner complex through interaction with the label or tag. An exemplary tag of this type is a poly-histidine sequence, generally around six histidine residues, that permits isolation of a compound so labeled using nickel chelation. Other labels and tags, such as the FLAG® tag (Eastman Kodak, Rochester, NY), well known and routinely used in the art, are embraced by the invention.

[00267] In one variation of an *in vitro* assay, the invention provides a method comprising the steps of (a) contacting an immobilized *M. tuberculosis* electron transport chain polypeptide with a candidate binding partner compound and (b) detecting binding of the candidate compound to the *M. tuberculosis* electron transport chain polypeptide. In an alternative embodiment, the candidate binding partner compound is immobilized and binding of *M. tuberculosis* electron transport chain polypeptide is detected. Immobilization is accomplished using any of the methods well known in the art, including covalent bonding to a support, a bead, or a chromatographic resin, as well as non-covalent, high affinity interactions such as antibody binding, or use of streptavidin/biotin binding wherein the immobilized compound includes a biotin moiety. Detection of binding can be accomplished (i) using a radioactive label on the compound that is not immobilized, (ii) using of a fluorescent label on the non-immobilized compound, (iii) using an antibody immunospecific for the non-immobilized compound, (iv) using a label on the non-immobilized compound that excites a fluorescent support to which the immobilized compound is attached, as well as other techniques well known and routinely practiced in the art.

[00268] The invention also provides cell-based assays to identify binding partner compounds of a *M. tuberculosis* electron transport chain polypeptide. In one embodiment, the invention provides a method comprising the steps of contacting a *M. tuberculosis* electron transport chain polypeptide expressed on the surface of a cell with a candidate binding partner compound and detecting binding of the candidate binding partner compound to the *M. tuberculosis* electron transport chain polypeptide. In a preferred embodiment, the detection comprises detecting a calcium flux or other physiological event in the cell caused by the binding of the molecule.

[00269] Another aspect of the present invention is directed to methods of identifying compounds that bind to either *M. tuberculosis* electron transport chain polypeptides or

nucleic acid molecules encoding *M. tuberculosis* electron transport chain polypeptides, comprising contacting *M. tuberculosis* electron transport chain polypeptides, or a nucleic acid molecule encoding the same, with a compound, and determining whether the compound binds *M. tuberculosis* electron transport chain polypeptides or nucleic acid molecules encoding the same. Binding can be determined by binding assays which are well known to the skilled artisan, including, but not limited to, gel-shift assays, Western blots, radiolabeled competition assay, phage-based expression cloning, co-fractionation by chromatography, co-precipitation, cross linking, interaction trap/two-hybrid analysis, southwestern analysis, ELISA, and the like, which are described in, for example, *Current Protocols in Molecular Biology*, 1999, John Wiley & Sons, NY, which is incorporated herein by reference in its entirety. The compounds to be screened include (which may include compounds which are suspected to bind *M. tuberculosis* electron transport chain polypeptides, or a nucleic acid molecule encoding the same), but are not limited to, extracellular, intracellular, biologic or chemical origin. The methods of the invention also embrace ligands that are attached to a label, such as a radiolabel (*e.g.*, ^{125}I, ^{35}S, ^{32}P, ^{33}P, ^{3}H), a fluorescence label, a chemiluminescent label, an enzymic label and an immunogenic label. The *M. tuberculosis* electron transport chain polypeptide or polynucleotide employed in such a test may either be free in solution, attached to a solid support, borne on a cell surface or located intracellularly or associated with a portion of a cell. One skilled in the art can, for example, measure the formation of complexes between *M. tuberculosis* electron transport chain polypeptides and the compound being tested. Alternatively, one skilled in the art can examine the diminution in complex formation between *M. tuberculosis* electron transport chain polypeptide and its substrate caused by the compound being tested.

[00270] In another embodiment of the invention, high throughput screening for compounds having suitable binding affinity to a *M. tuberculosis* electron transport chain polypeptide is employed. Briefly, large numbers of different test compounds are synthesized on a solid substrate. The peptide test compounds are contacted with *M. tuberculosis* electron transport chain polypeptides and washed. Bound *M. tuberculosis* electron transport chain polypeptide is then detected by methods well known in the art. Purified polypeptides of the invention can also be coated directly onto plates for use in the aforementioned drug screening techniques. In addition, non-neutralizing antibodies can be used to capture the protein and immobilize it on the solid support.

[00271] Generally, an expressed *M. tuberculosis* electron transport chain polypeptide can be used for HTS binding assays in conjunction with a defined partner. The identified peptide is labeled with a suitable radioisotope, including, but not limited to, ^{125}I, ^{3}H, ^{35}S or ^{32}P, by methods that are well known to those skilled in the art. Alternatively, the peptides may be labeled by well-known methods with a suitable fluorescent derivative (Baindur *et al.*, *Drug Dev. Res.*, 1994, *33*, 373-398; Rogers, *Drug Discovery Today*, 1997, *2*, 156-160). Radioactive ligand specifically bound to the protein in membrane preparations made from the cell line expressing the recombinant protein can be detected in HTS assays in one of several standard ways, including filtration of the protein-ligand complex to separate bound ligand from unbound ligand (Williams, *Med. Res. Rev.*, 1991, *11*, 147-184; Sweetnam *et al.*, *J. Natural Products*, 1993, *56*, 441-455). Alternative methods include a scintillation proximity assay (SPA) or a FlashPlate format in which such separation is unnecessary (Nakayama, *Cur. Opinion Drug Disc. Dev.*, 1998, *1*, 85-91 Bossé *et al.*, *J. Biomolecular Screening*, 1998, *3*, 285-292.). Binding of fluorescent ligands can be detected in various ways, including fluorescence energy transfer (FRET), direct spectrophotofluorometric analysis of bound ligand, or fluorescence polarization (Rogers, *Drug Discovery Today*, 1997, *2*, 156-160; Hill, *Cur. Opinion Drug Disc. Dev.*, 1998, *1*, 92-97).

[00272] Other assays may be used to identify specific partners of a *M. tuberculosis* electron transport chain polypeptide, including assays that identify partners of the target protein through measuring direct binding of test partners to the target protein, as well as assays that identify partners of target proteins through affinity ultrafiltration with ion spray mass spectroscopy/HPLC methods or other physical and analytical methods. Alternatively, such binding interactions are evaluated indirectly using the yeast two-hybrid system described in Fields *et al.*, Nature, 340:245-246 (1989), and Fields *et al.*, Trends in Genetics, 10:286-292 (1994), both of which are incorporated herein by reference. The two-hybrid system is a genetic assay for detecting interactions between two proteins or polypeptides. It can be used to identify proteins that bind to a known protein of interest, or to delineate domains or residues critical for an interaction. Variations on this methodology have been developed to clone genes that encode DNA binding proteins, to identify peptides that bind to a protein, and to screen for drugs. The two-hybrid system exploits the ability of a pair of interacting proteins to bring a transcription activation domain into close proximity with a DNA binding domain that binds to an upstream activation sequence (UAS) of a reporter gene, and is

generally performed in yeast. The assay requires the construction of two hybrid genes encoding (1) a DNA-binding domain that is fused to a first protein and (2) an activation domain fused to a second protein. The DNA-binding domain targets the first hybrid protein to the UAS of the reporter gene; however, because most proteins lack an activation domain, this DNA-binding hybrid protein does not activate transcription of the reporter gene. The second hybrid protein, which contains the activation domain, cannot by itself activate expression of the reporter gene because it does not bind the UAS. However, when both hybrid proteins are present, the noncovalent interaction of the first and second proteins tethers the activation domain to the UAS, activating transcription of the reporter gene. For example, when the first protein is a Rel gene product, or fragment thereof, that is known to interact with another protein or nucleic acid, this assay can be used to detect agents that interfere with the binding interaction. Expression of the reporter gene is monitored as different test agents are added to the system. The presence of an inhibitory agent results in lack of a reporter signal.

[00273] The yeast two-hybrid assay can also be used to identify proteins that bind to the gene product. In an assay to identify proteins that bind to a *M. tuberculosis* electron transport chain polypeptide, or fragment thereof, a fusion polynucleotide encoding both a *M. tuberculosis* electron transport chain polypeptide (or fragment) and a UAS binding domain (*i.e.*, a first protein) may be used. In addition, a large number of hybrid genes each encoding a different second protein fused to an activation domain are produced and screened in the assay. Typically, the second protein is encoded by one or more members of a total cDNA or genomic DNA fusion library, with each second protein-coding region being fused to the activation domain. This system is applicable to a wide variety of proteins, and it is not even necessary to know the identity or function of the second binding protein. The system is highly sensitive and can detect interactions not revealed by other methods; even transient interactions may trigger transcription to produce a stable mRNA that can be repeatedly translated to yield the reporter protein.

[00274] Other assays may be used to search for agents that bind to the target protein. One such screening method to identify direct binding of test ligands to a target protein is described in U.S. Patent No. 5,585,277, incorporated herein by reference. This method relies on the principle that proteins generally exist as a mixture of folded and unfolded states, and continually alternate between the two states. When a test ligand binds to the folded form of a

target protein (*i.e.*, when the test ligand is a ligand of the target protein), the target protein molecule bound by the ligand remains in its folded state. Thus, the folded target protein is present to a greater extent in the presence of a test ligand which binds the target protein, than in the absence of a ligand. Binding of the ligand to the target protein can be determined by any method that distinguishes between the folded and unfolded states of the target protein. The function of the target protein need not be known in order for this assay to be performed. Virtually any agent can be assessed by this method as a test ligand, including, but not limited to, metals, polypeptides, proteins, lipids, polysaccharides, polynucleotides and small organic molecules.

[00275] Another method for identifying ligands of a target protein is described in Wieboldt *et al.*, Anal. Chem., 69:1683-1691 (1997), incorporated herein by reference. This technique screens combinatorial libraries of 20-30 agents at a time in solution phase for binding to the target protein. Agents that bind to the target protein are separated from other library components by simple membrane washing. The specifically selected molecules that are retained on the filter are subsequently liberated from the target protein and analyzed by HPLC and pneumatically assisted electrospray (ion spray) ionization mass spectroscopy. This procedure selects library components with the greatest affinity for the target protein, and is particularly useful for small molecule libraries.

[00276] Other embodiments of the invention comprise using competitive screening assays in which neutralizing antibodies capable of binding a polypeptide of the invention specifically compete with a test compound for binding to the polypeptide. In this manner, the antibodies can be used to detect the presence of any peptide that shares one or more antigenic determinants with *M. tuberculosis* electron transport chain polypeptides. Radiolabeled competitive binding studies are described in A.H. Lin *et al. Antimicrobial Agents and Chemotherapy*, 1997, vol. 41, no. 10. pp. 2127-2131, the disclosure of which is incorporated herein by reference in its entirety.

Identification of modulating agents

[00277] The invention also provides methods for identifying a modulator of binding between a *M. tuberculosis* electron transport chain polypeptide and a binding partner thereof, comprising the steps of: (a) contacting a *M. tuberculosis* electron transport chain polypeptide binding partner and a composition comprising a *M. tuberculosis* electron transport chain polypeptide in the presence and in the absence of a putative modulator compound; (b)

detecting binding between the binding partner and the *M. tuberculosis* electron transport chain polypeptide; and (c) identifying a putative modulator compound or a modulator compound in view of decreased or increased binding between the binding partner and the *M. tuberculosis* electron transport chain polypeptide in the presence of the putative modulator, as compared to binding in the absence of the putative modulator. Following steps (a) and (b), compounds identified as modulating binding between *M. tuberculosis* electron transport chain polypeptides and its binding partner may be tested in other assays including, but not limited to, *in vivo* models, to confirm or quantitate modulation of binding to *M. tuberculosis* electron transport chain polypeptides.

[00278] *M. tuberculosis* electron transport chain polypeptide binding partners that stimulate electron transport chain activity are useful as agonists in disease states or conditions characterized by insufficient electron transport chain activity (*e.g.*, as a result of insufficient activity of a ligand of the electron transport chain). In addition *M. tuberculosis* electron transport chain polypeptide modulators in general, as well as *M. tuberculosis* electron transport chain polynucleotides and polypeptides, are useful in diagnostic assays for such diseases or conditions.

[00279] In another aspect, the invention provides methods for treating a disease or abnormal condition by administering to a patient in need of such treatment a substance that modulates the activity or expression of a polypeptide having sequences of SEQ ID NOs:1, 3, 5, 7, 9 or 11.

[00280] Agents that modulate *M. tuberculosis* electron transport chain polypeptide activity or expression may be identified by incubating a putative modulator with a cell containing a *M. tuberculosis* electron transport chain polypeptide or polynucleotide and determining the effect of the putative modulator on *M. tuberculosis* electron transport chain polypeptide activity or expression. The selectivity of a compound that modulates the activity of *M. tuberculosis* electron transport chain polypeptides can be evaluated by comparing its effects on *M. tuberculosis* electron transport chain polypeptides to its effect on other related proteins. Following identification of compounds that modulate *M. tuberculosis* electron transport chain polypeptide activity or expression, such compounds may be further tested in other assays including, but not limited to, *in vivo* models, in order to confirm or quantitate their activity. Selective modulators may include, for example, antibodies and other proteins, peptides, or organic molecules that specifically bind to a *M. tuberculosis* electron transport chain

polypeptide or a *M. tuberculosis* electron transport chain polypeptide-encoding nucleic acid. Modulators of *M. tuberculosis* electron transport chain polypeptide activity will be therapeutically useful in treatment of diseases and physiological conditions in which normal or aberrant *M. tuberculosis* electron transport chain polypeptide activity is involved. *M. tuberculosis* electron transport chain polynucleotides, polypeptides, and modulators may be used in the treatment of such diseases and conditions as infections, such as tuberculosis, staph, strep, and other bacterial infections.

[00281] Methods of the invention to identify modulators include variations on any of the methods described above to identify binding partner compounds, the variations including techniques wherein a binding partner compound has been identified and the binding assay is carried out in the presence and absence of a candidate modulator. A modulator is identified in those instances where binding between the *M. tuberculosis* electron transport chain polypeptide and the binding partner compound changes in the presence of the candidate modulator compared to binding in the absence of the candidate modulator compound. A modulator that increases binding between the *M. tuberculosis* electron transport chain polypeptide and the binding partner compound is described as an enhancer or activator, and a modulator that decreases binding between the *M. tuberculosis* electron transport chain polypeptide and the binding partner compound is described as an inhibitor. Following identification of modulators, such compounds may be further tested in other assays including, but not limited to, *in vivo* models, in order to confirm or quantitate their activity as modulators.

[00282] The invention also comprehends high-throughput screening (HTS) assays to identify compounds that interact with or inhibit biological activity (*i.e.*, affect enzymatic activity, binding activity, *etc.*) of a *M. tuberculosis* electron transport chain polypeptide. HTS assays permit screening of large numbers of compounds in an efficient manner. Cell-based HTS systems are contemplated to investigate *M. tuberculosis* electron transport chain polypeptide-ligand interaction. HTS assays are designed to identify "hits" or "lead compounds" having the desired property, from which modifications can be designed to improve the desired property. Chemical modification of the "hit" or "lead compound" is often based on an identifiable structure/activity relationship between the "hit" and the *M. tuberculosis* electron transport chain polypeptide.

[00283] Another aspect of the present invention is directed to methods of identifying compounds which modulate (*i.e.*, increase or decrease) an activity of *M. tuberculosis* electron transport chain polypeptides comprising contacting a *M. tuberculosis* electron transport chain polypeptides with a compound, and determining whether the compound modifies activity of the *M. tuberculosis* electron transport chain polypeptide. The activity in the presence of the test compared is measured to the activity in the absence of the test compound. Where the activity of the sample containing the test compound is higher than the activity in the sample lacking the test compound, the compound will have increased activity. Similarly, where the activity of the sample containing the test compound is lower than the activity in the sample lacking the test compound, the compound will have inhibited activity. Following the identification of compounds that modulate an activity of a *M. tuberculosis* electron transport chain polypeptide, such compounds can be further tested in other assays including, but not limited to, *in vivo* models, in order to confirm or quantitate their activity.

[00284] The present invention is particularly useful for screening compounds by using *M. tuberculosis* electron transport chain polypeptides in any of a variety of drug screening techniques. The compounds to be screened include (which may include compounds which are suspected to modulate *M. tuberculosis* electron transport chain polypeptide activity), but are not limited to, extracellular, intracellular, biologic or chemical origin. The *M. tuberculosis* electron transport chain polypeptide employed in such a test may be in any form, preferably, free in solution, attached to a solid support, borne on a cell surface or located intracellularly. One skilled in the art can, for example, measure the formation of complexes between *M. tuberculosis* electron transport chain polypeptides and the compound being tested. Alternatively, one skilled in the art can examine the diminution in complex formation between a *M. tuberculosis* electron transport chain polypeptide and its substrate caused by the compound being tested. In some embodiments, fragments comprising one or more functional domains of *M. tuberculosis* electron transport chain polypeptides are used.

[00285] The activity of *M. tuberculosis* electron transport chain polypeptides of the invention can be determined by, for example, examining the ability to hydrolyze or synthesize (p)ppGpp.

[00286] The modulators of the invention exhibit a variety of chemical structures, which can be generally grouped into non-peptide mimetics of natural *M. tuberculosis* electron transport chain polypeptide ligands, peptide and non-peptide allosteric effectors of Rel protein, and

peptides that may function as activators or inhibitors (competitive, uncompetitive and non-competitive) (*e.g.*, antibody products) of *M. tuberculosis* electron transport chain polypeptides. The invention does not restrict the sources for suitable modulators, which may be obtained from natural sources such as plant, animal or mineral extracts, or non-natural sources such as small molecule libraries, including the products of combinatorial chemical approaches to library construction, and peptide libraries.

[00287] Other assays can be used to examine enzymatic activity including, but not limited to, photometric, radiometric, HPLC, electrochemical, and the like, which are described in, for example, *Enzyme Assays: A Practical Approach*, eds. R. Eisenthal and M. J. Danson, **1992**, Oxford University Press, which is incorporated herein by reference in its entirety.

[00288] The use of cDNAs encoding proteins in drug discovery programs is well known; assays capable of testing thousands of unknown compounds per day in high-throughput screens (HTSs) are thoroughly documented. The literature is replete with examples of the use of radiolabeled ligands in HTS binding assays for drug discovery (see Williams, *Medicinal Research Reviews*, **1991**, *11*, 147-184.; Sweetnam, *et al.*, *J. Natural Products*, **1993**, *56*, 441-455 for review). Recombinant proteins are preferred for binding assay HTS because they allow for better specificity (higher relative purity), provide the ability to generate large amounts of protein material, and can be used in a broad variety of formats (see Hodgson, *Bio/Technology*, **1992**, *10*, 973-980; each of which is incorporated herein by reference in its entirety).

[00289] A variety of heterologous systems is available for functional expression of recombinant proteins that are well known to those skilled in the art. Such systems include bacteria (Strosberg, *et al.*, *Trends in Pharmacological Sciences*, **1992**, *13*, 95-98), yeast (Pausch, *Trends in Biotechnology*, **1997**, *15*, 487-494), several kinds of insect cells (Vanden Broeck, *Int. Rev. Cytology*, **1996**, *164*, 189-268), amphibian cells (Jayawickreme *et al.*, *Current Opinion in Biotechnology*, **1997**, *8*, 629-634) and several mammalian cell lines (CHO, HEK-293, COS, etc.; see Gerhardt, *et al.*, *Eur. J. Pharmacology*, **1997**, *334*, 1-23). These examples do not preclude the use of other possible cell expression systems, including cell lines obtained from nematodes (PCT application WO 98/37177).

[00290] In some embodiments of the invention, methods of screening for compounds that modulate *M. tuberculosis* electron transport chain polypeptide activity comprise contacting test compounds with a *M. tuberculosis* electron transport chain polypeptide and assaying for

the presence of a complex between the compound and the *M. tuberculosis* electron transport chain polypeptide. In such assays, the ligand is typically labeled. After suitable incubation, free ligand is separated from that present in bound form, and the amount of free or uncomplexed label is a measure of the ability of the particular compound to bind to the *M. tuberculosis* electron transport chain polypeptide.

[00291] Candidate modulators contemplated by the invention include compounds selected from libraries of either potential activators or potential inhibitors. There are a number of different libraries used for the identification of small molecule modulators, including: (1) chemical libraries, (2) natural product libraries, and (3) combinatorial libraries comprised of random peptides, oligonucleotides or organic molecules. Chemical libraries consist of random chemical structures, some of which are analogs of known compounds or analogs of compounds that have been identified as "hits" or "leads" in other drug discovery screens, some of which are derived from natural products, and some of which arise from non-directed synthetic organic chemistry. Natural product libraries are collections of microorganisms, animals, plants, or marine organisms which are used to create mixtures for screening by: (1) fermentation and extraction of broths from soil, plant or marine microorganisms or (2) extraction of plants or marine organisms. Natural product libraries include polyketides, non-ribosomal peptides, and variants (non-naturally occurring) thereof. For a review, see Science 282:63-68 (1998). Combinatorial libraries are composed of large numbers of peptides, oligonucleotides, or organic compounds as a mixture. These libraries are relatively easy to prepare by traditional automated synthesis methods, PCR, cloning, or proprietary synthetic methods. Of particular interest are non-peptide combinatorial libraries. Still other libraries of interest include peptide, protein, peptidomimetic, multiparallel synthetic collection, recombinatorial, and polypeptide libraries. For a review of combinatorial chemistry and libraries created therefrom, see Myers, Curr. Opin. Biotechnol. 8:701-707 (1997). Identification of modulators through use of the various libraries described herein permits modification of the candidate "hit" (or "lead") to optimize the capacity of the "hit" to modulate activity.

[00292] Still other candidate inhibitors contemplated by the invention can be designed and include soluble forms of binding partners, as well as such binding partners as chimeric, or fusion, proteins. A "binding partner" as used herein broadly encompasses non-peptide modulators, as well as such peptide modulators as neuropeptides other than natural ligands,

antibodies, antibody fragments, and modified compounds comprising antibody domains that are immunospecific for the expression product of the identified *M. tuberculosis* electron transport chain genes.

[00293] The polypeptides of the invention are also employed as a research tool for identification, characterization and purification of interacting, regulatory proteins. Appropriate labels are incorporated into the polypeptides of the invention by various methods known in the art and the polypeptides are used to capture interacting molecules. For example, molecules are incubated with the labeled polypeptides, washed to remove unbound polypeptides, and the polypeptide complex is quantified. Data obtained using different concentrations of polypeptide are used to calculate values for the number, affinity, and association of polypeptide with the protein complex.

[00294] Labeled polypeptides are also useful as reagents for the purification of molecules with which the polypeptide interacts including, but not limited to, inhibitors. In one embodiment of affinity purification, a polypeptide is covalently coupled to a chromatography column. Cells and their membranes are extracted, and various cellular subcomponents are passed over the column. Molecules bind to the column by virtue of their affinity to the polypeptide. The polypeptide-complex is recovered from the column, dissociated and the recovered molecule is subjected to protein sequencing. This amino acid sequence is then used to identify the captured molecule or to design degenerate oligonucleotides for cloning the corresponding gene from an appropriate cDNA library.

[00295] Alternatively, compounds may be identified which exhibit similar properties to the ligands for the *M. tuberculosis* electron transport chain polypeptides of the invention, but which are smaller and exhibit a longer half time than the endogenous ligand in a human or animal body. When an organic compound is designed, a molecule according to the invention is used as a "lead" compound. The design of mimetics to known pharmaceutically active compounds is a well-known approach in the development of pharmaceuticals based on such "lead" compounds. Mimetic design, synthesis and testing are generally used to avoid randomly screening a large number of molecules for a target property. Furthermore, structural data deriving from the analysis of the deduced amino acid sequences encoded by the DNAs of the present invention are useful to design new drugs, more specific and therefore with a higher pharmacological potency.

[00296] In some embodiments, the novel molecules identified by the screening methods according to the invention are low molecular weight organic molecules, in which case a composition or pharmaceutical composition can be prepared thereof for oral intake, such as in tablets. The compositions, or pharmaceutical compositions, comprising the nucleic acid molecules, vectors, polypeptides, antibodies and compounds identified by the screening methods described herein, can be prepared for any route of administration including, but not limited to, oral, intravenous, cutaneous, subcutaneous, nasal, intramuscular or intraperitoneal. The nature of the carrier or other ingredients will depend on the specific route of administration and particular embodiment of the invention to be administered. Examples of techniques and protocols that are useful in this context are, *inter alia*, found in Remington's Pharmaceutical Sciences, 16th edition, Osol, A (ed.), **1980**, which is incorporated herein by reference in its entirety.

[00297] The dosage of these low molecular weight compounds will depend on the disease state or condition to be treated and other clinical factors such as weight and condition of the human or animal and the route of administration of the compound. For treating human or animals, between approximately 0.5 mg/kg of body weight to 500 mg/kg of body weight of the compound can be administered. Therapy is typically administered at lower dosages and is continued until the desired therapeutic outcome is observed.

[00298] The present compounds and methods, including nucleic acid molecules, polypeptides, antibodies, compounds identified by the screening methods described herein, have a variety of pharmaceutical applications and may be used, for example, to treat or prevent unregulated cellular growth, such as cancer cell and tumor growth. In a particular embodiment, the present molecules are used in gene therapy. For a review of gene therapy procedures, see *e.g.* Anderson, *Science*, 1992, *256*, 808-813, which is incorporated herein by reference in its entirety.

[00299] The present invention also encompasses methods of agonizing (stimulating) or antagonizing a *M. tuberculosis* electron transport chain polypeptide natural binding partner associated activity in a mammal comprising administering to said mammal an agonist or antagonist to one of the above disclosed polypeptides in an amount sufficient to effect said agonism or antagonism. One embodiment of the present invention, then, is a method of treating diseases in a mammal with an agonist or antagonist of the protein of the present invention comprises administering the agonist or antagonist to a mammal in an amount

sufficient to agonize or antagonize *M. tuberculosis* electron transport chain-associated functions.

[00300] Methods of determining the dosages of compounds to be administered to a patient and modes of administering compounds to an organism are disclosed in International patent publication number WO 96/22976, published August 1 1996, which is incorporated herein by reference in its entirety, including any drawings, figures or tables. Those skilled in the art will appreciate that such descriptions are applicable to the present invention and can be easily adapted to it.

[00301] The proper dosage depends on various factors such as the type of disease being treated, the particular composition being used and the size and physiological condition of the patient. Therapeutically effective doses for the compounds described herein can be estimated initially from cell culture and animal models. For example, a dose can be formulated in animal models to achieve a circulating concentration range that initially takes into account the IC_{50} as determined in cell culture assays. The animal model data can be used to more accurately determine useful doses in humans.

[00302] Plasma half-life and biodistribution of the drug and metabolites in the plasma, tumors and major organs can also be determined to facilitate the selection of drugs most appropriate to inhibit a disorder. Such measurements can be carried out. For example, HPLC analysis can be performed on the plasma of animals treated with the drug and the location of radiolabeled compounds can be determined using detection methods such as X-ray, CAT scan and MRI. Compounds that show potent inhibitory activity in the screening assays, but have poor pharmacokinetic characteristics, can be optimized by altering the chemical structure and retesting. In this regard, compounds displaying good pharmacokinetic characteristics can be used as a model.

[00303] Toxicity studies can also be carried out by measuring the blood cell composition. For example, toxicity studies can be carried out in a suitable animal model as follows: 1) the compound is administered to mice (an untreated control mouse should also be used); 2) blood samples are periodically obtained via the tail vein from one mouse in each treatment group; and 3) the samples are analyzed for red and white blood cell counts, blood cell composition and the percent of lymphocytes versus polymorphonuclear cells. A comparison of results for each dosing regime with the controls indicates if toxicity is present.

[00304] At the termination of each toxicity study, further studies can be carried out by sacrificing the animals (preferably, in accordance with the American Veterinary Medical Association guidelines Report of the American Veterinary Medical Assoc. Panel on Euthanasia, Journal of American Veterinary Medical Assoc., 202:229-249, 1993). Representative animals from each treatment group can then be examined by gross necropsy for immediate evidence of metastasis, unusual illness or toxicity. Gross abnormalities in tissue are noted and tissues are examined histologically. Compounds causing a reduction in body weight or blood components are less preferred, as are compounds having an adverse effect on major organs. In general, the greater the adverse effect the less preferred the compound.

Methods of detecting the presence of *M. tuberculosis*

[00305] The present invention also provides methods for detecting the presence of *M. tuberculosis* in a sample comprising: contacting the sample with an electron transport chain inhibitor comprising a detectable label and detecting evidence of the electron transport chain inhibitor in said sample, wherein evidence of the electron transport chain inhibitor is indicative of the presence of *M. tuberculosis*. In some embodiments the sample is a human sample. In some embodiments the method further comprises comparing the results of said contacting with a control.

[00306] The following examples are meant to illustrate the invention and are not to be construed to limit the invention in any way. Those skilled in the art will recognize modifications that are within the spirit and scope of the invention.

Examples

Example 1. Spectroscopy

[00307] Aerobically grown, gamma irradiated *Mycobacterium tuberculosis* $H_{37}Rv$ whole cells were obtained. Cells (12 g wet weight) were washed with 10 mL of phopshate-digitonin buffer (50 mM H_2PO_4, 0.1% digitonin [pH 7.4]) and resuspended in the same buffer. The bacteria were lysed by passage through a French press at 14,000 kPa and the product was centrifuged at 12,000 × *g* for 15 min to remove cellular debris. The supernatant was further clarified by centrifugation at 50,000 × *g* for 50 min. The ultra-centrifuged supernatant was used as the membrane-containing fraction for spectral studies. To isolate cell membranes, the

50,000 × *g* supernatant was centrifuged at 150,000 × *g* for 60 min. The resulting pellet was washed with buffer, resuspended, then centrifuged at 150,000 × *g* for 60 min a second time. Protein concentrations were determined by bicinchoninic assay (BCA; Pierce) using bovine serum albumin as the standard. Spectra were recorded at room temperature on a Cary 4E dual beam spectrophotometer, using a scan speed of 120 nm/min and a slit width of 1 nm. Reduction of 21.3 mg/ml membrane protein was accomplished by a 3 minute incubation with 5 mM NADH. To illustrate the spectral perturbation due to carbon monoxide binding of terminal oxidases, 100 μM CO was added prior to NADH reduction. For the samples containing TPZ, drug was added to the sample and incubated for 5 minutes before the addition of NADH.

Example 2. Amperometric Assay

[00308] Oxygen uptake in the presence of trifluoperazine was measured by a polarographic assay as per the Smith-Davies method. *M. tuberculosis* membrane (50 mg/ml) was added to 0.1 M phosphate buffer (pH 7.4) at the time indicated by the first arrow. The second arrow in the traces indicates the addition of either 1 mM trifluoperazine or vehicle. The addition of 1 mM trifluoperazine inhibited respiration 94% within 40 seconds, which is comparable to the speed and magnitude of 10 mM potassium cyanide action. Oxygen uptake was restored with the addition of 10 mM ascorbate and 1 mM TMPD (Sigma-Aldrich, St. Louis, MO).

Example 3. NADH:Menaquinone oxidoreductase Assay

[00309] *M. tuberculosis* membrane protein was dialysed overnight in phosphate buffer (100 mM KH_2PO_4, pH 7.5, 1 mM EDTA, 4° C) to remove endogenous reducing agents. The transfer of electrons from NADH to menaquinone-1 (MQ_1) was determined by monitoring the oxidation of NADH using a custom built Hitachi1098 spectrophotometer. *MTb* membrane protein (3 mg) was diluted into 1.1 mL phosphate buffer supplemented with 10 mM KCN and 100 uM MQ_1 (20 mM stock in ethanol). Drug or vehicle was added as desired and the reaction mixture was incubated for 5 minutes at 30°C. The experiment was initiated upon the addition of 100 uM NADH and the decrease in light absorbance at 340 nm was followed on a chart recorder. NADH was not oxidized in the absence of membrane protein or MQ_1.

Example 4. Determination of the MIC by the BACTEC MGIT 960.

[00310] The MIC for each compound was determined by the Bactec MGIT 960 system. $H_{37}R_v$ *M. tuberculosis* (gift of Dr. Jean Patel, University of Pennsylvania, Philadelphia, PA) was grown in Middlebrook 7H9 broth until the growth index (GI) reached 75 and was then diluted 2500 fold and used as the inocula. The vials were dispensed with different dilutions of drug to reach final concentrations ranging from 0.2 to 26 μg/ml. All of the drug-containing vials were inoculated with 0.5 ml of the bacterial suspensions prepared as described above. Six drug-free controls were included with each test: three were inoculated with 0.5 ml of the suspension, and the remaining three were inoculated with 0.5 ml of a 1/100 dilution of the suspension. The vials were incubated at 37°C and were read in a BACTEC 960 reader (Becton Dickinson, Sparks, MD) every day until the GI in the 1/100 diluted control reached 75, with an increase in the GI of at least 10 for 3 consecutive days. The time to positive was 10 days for the undiluted control. MIC was defined as the lowest concentration of the drug that caused an increase in the GI equal to or less than the increase in the GI of the control diluted 1/100.

Example 5 Inhibitors of Mycobacteria

Mycobacterial Growth is Inhibited By Chlorpromazine And Cyanide, But is Resistant to Mitochondrial Inhibitors.

[00311] In *Mycobacterium smegmatis*, chlorpromazine was as potent an inhibitor of growth as potassium cyanide (Figure 4). Similar to many microorganisms, it demonstrated resistance to mitochondrial inhibitors antimycin and myxothiazol, which were effective only at near saturating concentrations. Resistance to well known respiratory inhibitors does reinforce the notion that the *MTb* respiratory components are significantly different than those of mitochondria.

Chlorpromazine Inhibits The Growth Of *M. Smegmatis* Dose Dependently

[00312] The dose response relationship of chlorpromazine inhibition of *Mycobacterium smegmatis* growth was assayed (Figure 5). Inhibition by CPZ concentrations as low as 10 μM was noted in the experimental conditions. Drug susceptibility testing can also be performed in a fermentation system with controlled oxygen tension to determine the extent of

inhibition under various environmental conditions such as, for example, reduced oxygen tension.

Chlorpromazine Inhibits Respiration Of *M. Smegmatis*

[00313] Oxygen uptake in the presence of chlorpromazine was measured using a polarographic assay as described by Smith et al (1979). *M. smegmatis* was (50 mg/ml) was added to 0.1 M phosphate buffer at the time indicated by the first arrow (Figure 6). The second arrow in the traces indicated the addition of phosphate buffer (Figure 6A) or 1 mM chlorpromazine (Figure 6B). The addition chlorpromazine inhibited respiration 94% within 40 seconds, which is comparable to the speed and magnitude of potassium cyanide action (data not shown).

Example 6. High Throughput Screening

[00314] Analogs of chlorpromazine are tested for antitubercular activity by a high throughput screen. A soluble redox dye system provides a rapid, quantitative measure of chemosensitivity without the risks involved in using live organisms. Tetrazolium salts were developed nearly 50 years ago as a colorimetric assay of cell growth (Black and Speer, 1954, Bernebei et al., 1989). The technique has been subsequently refined to a semiautomated system and has been successfully extended to immunological cytotoxicity assays (Denizot and Lang, 1986) and tumor cell line chemosensitivity testing (Carmichael, 1987). A commercial kit is available from Promega (CellTiter 96™ AQueous cell proliferation assay).

[00315] In a sample assay, *MTb* electron transport particles are added to a 96 well plate. Following an incubation period, dye is added, and a second incubation period allows formazan development. Samples are read at 1-4 hours using an ELISA plate reader at 490nm. The amount of color produced is directly proportional to the reducing activity of the suspension. A drug that blocks electron transport will block the formation of the colored formazan product (Slater, 1963, Lippold, 1982).

[00316] In some embodiments, with added controls, up to 47 compounds are tested per plate. The assay may be further refined through the choice of Tetrazolium compound. A number of tetrazolium salts are available with a wide range of reduction potentials (Table 1).

Table 1: Commercially available tetrazolium salts

Dye	Reduction Potential (V) (Altman, 1976)	Extinction Maximum (nm) (Lippold, 1982)
Nitroblue tetratzolium (NBT)	0.05	730
Iodo-nitro-tetrazolium (INT)	0.09	640
Dimethyl thiazolyly tetrazolium (MTT)	0.11	500
Blue Tetrazolium (BT)	0.16	580
Tetrazolium Red (TT)	0.49	488

Example 7. The Electron Transport Pathway as a Target for New Drug Development

[00317] *MTb ndh* and *ndhA* have been cloned from genomic *MTb* DNA and inserted into a PET15b plasmid, thus conferring a hexa-histidine upstream sequence. The gene integrity was confirmed by sequencing and the subsequent expressed protein was purified in two chromatographic steps (Figures 7A and 7B). Activity assays were performed, and data has been obtained showing direct inhibition of the purified type II NADH:menaquinone oxidoreductase using chlorpromazine and trifluperazine, as well as a mechanism of action of the drugs- the inhibition of the binding of the cofactor FAD.

[00318] Activity assays were performed spectroscopically by noting the decrease in absorbance of NADH at 340 nm. Results indicated that the recombinant enzyme (type II NADH:quinone oxidoreductase) was capable of oxidizing NADH only in the presence of ubiquinone-1 (Q_1) and flavin adenine dinucleotide (FAD). The rate of oxidation of NADH in the absence of either substrate (ubiquinone-1 [Q1]) or FAD prevented the transfer of electrons. Phenothiazines (TPZ and CPZ) inhibited the enzyme with the same relative potency as observed *in vitro* against *MTb* growth (see Figures 8A-C).

Example 8

[00319] Actively growing *Mycobacterium tuberculosis* bacilli have an obligate requirement for oxygen; exposure to anaerobic conditions produces a state of repressed growth. Given the critical role of oxygen in the generation of cellular energy and long term survival little

information is available on oxidative phosphorylation in *M. tuberculosis*. Analysis of the *MTb* genome indicates the presence of a respiratory chain with two major branches, a quinol oxidase branch and a cytochrome oxidase branch (Figure 1). Cytochrome *bd* oxidase is a quinol oxidase encoded by *cydABCD* genes, and cytochrome aa_3 is a cytochrome *c* oxidase encoded by *ctaBCDE* genes. The *d*-type oxidase is induced in mycobacteria under low oxygen conditions and is important for microaerobic growth. The *a*-type oxidase appears to belong to the well-characterized heme-copper oxidase superfamily of respiratory oxidases critical for exponential growth under oxygen rich conditions. The first subunit of the *a*-type oxidase, *ctaD*, shares 40% identity in a 511 amino acid overlap to the first subunit of mitochondrial cytochrome aa_3 and contains a putative copper center signature. *MTb* membrane preparations readily oxidize reduced bovine and yeast cytochrome *c*, yet extraction of *MTb* membranes under high salt conditions failed to release free cytochrome *c* (unpublished results). Inspection of the genome indicates that cytochrome *c* is fused to the third subunit (*qcrC*) of the bc_1 complex; *qcrC* shares 55% identity with the newly identified cytochrome cc_1 of *Corynebacterium glutamicum*[8] and contains two CXYCH cytochrome *c* binding motifs. It thus appears that *MTb* is a second member of a new clade of G+C rich gram positive organisms with a di-heme cytochrome c_1 subunit in the bc_1 complex. The physiologic electron donor to the bc_1 complex is demethylmenaquinone-8 (DMK-8) presumably synthesized by the enzymes encoded by the *menABCDEG* genes. The quinone pool is reduced by various oxidoreductases, including succinate:menaquinone oxidoreductase (complex II) which is composed of 4 subunits (*sdhABCD*) and NADH:menaquinone oxidoreductases. Two different types of NADH:menaquinone oxidoreductases are encoded in the *MTb* genome. The first is a type I NADH:menaquinone oxidoreductase (NDH-I), a 14 subunit (*nuoABCDEFGHIJKLMN*) complex that is presumably coupled to proton translocation, and the second is an alternative, non-proton pumping, single subunit type II NADH:menaquinone oxidoreductase (NDH-II), present in two copies (*ndh*, *ndhA*). The alternative *MTb* NADH:menaquinone oxidoreducatases share 67% sequence identity among themselves and their genes are located 17 Kb apart on the chromosome. Other mycobacterial species, however, such as *M. leprae* and *M. smegmatis* only contain single copies of the *ndh* gene with 91% and 83% identical residues respectively compared to *MTb ndh*. Furthermore, *M. leprae* is solely dependent upon *ndh* activity; all type I genes except a *nuoN* pseudogene have been lost in the course of evolution. It thus appears that type II NADH oxidoreductase

activity is of greater importance to the survival of mycobacteria. Indeed, inactivating mutations in *M. smegmatis ndh* lead to a thermosensitive lethal and auxotrophic phenotype. In *E. coli*, type II NADH oxidoreductase is dominant under conditions of aerobic growth, whereas type I NADH oxidoreductase is induced under anaerobic conditions. Therefore, type II NADH oxidoreductase is likely to be an important respiratory enzyme in *M. tuberculosis* for active, aerobic growth.

Example 9

[00320] To characterize the cytochromes corresponding to the bc_1 complex and terminal oxidases of *MTb*, we collected NADH-reduced minus air-oxidized difference spectra (Figure 2A) of *MTb* membrane particles. Peaks were observed at 563 nm, 532 nm, and 430 nm which are characteristic of the *b*- type cytochromes[14,15]. Also visible were peaks at 552 nm and 522 nm representing cytochrome *c* of the bc_1 complex. A broad peak at 600 nm with a resonance peak at 444 nm corresponded to cytochrome aa_3. The bacilli were grown under fully aerobic conditions, making the discrete *bd* oxidase peak at 432 nm difficult to discern.[6] Carbon monoxide binds to terminal oxidases, and thereby causes a shift in heme absorbance. To confirm that cytochrome aa_3 is a terminal oxidase, [NADH reduced plus carbon monoxide] minus [NADH reduced] spectra were obtained (Figure 2A). As expected, CO binding produced a trough at 444 nm and a peak at 429 nm. The cytochrome *c* peak of the bc_1 complex at 552 nm was not visible in the [NADH reduced plus carbon monoxide] minus [NADH reduced] difference spectrum, which is consistent with a cytochrome that does not bind oxygen.

[00321] The steady state rate of respiration of *MTb* membrane particles was determined in a cell-free amperometric assay. We added various respiratory inhibitors to observe the relative contribution of each branch of the chain to the overall respiratory rate. *MTb* membrane particles were resistant to the bc_1 complex inhibitors, antimycin and myxothiazol (IC_{50} 160 µM antimycin and 99 µM myxothiazol). However, work by Wong & Maier[16] indicated that the phenothiazine, chlorpromazine, inhibited electron transport between cytochromes *c* and a_1 + *o* (cytochrome *c* oxidase) in *Azotobacter vinelandii*. We therefore tested a series of phenothiazines, including trifluoperazine, as alternative inhibitors. Addition of NADH to *MTb* membrane particles resulted in an immediate linear consumption of oxygen, but was inhibited 100% by the addition of 1 mM of trifluoperazine. Respiration in the drug-arrested

membranes was restored by the addition of 10 mM ascorbate and 1 mM 3,3,5,5-tetramethylphenylenediamine (TMPD), which donates electrons at the level of cytochrome *c* (Figure 2B). Thus, the phenothiazine affected both of the respiratory branches in *MTb*. The recovery of oxygen consumption implied that the site of inhibition by trifluoperazine is upstream of cytochrome *c*. To confirm this, we tested the ability of trifluoperazine to inhibit the reduction of bovine cytochrome *c*. Using the 550 nm – 540 nm wavelength pair, we determined that trifluoperazine (100 μM) inhibited the rate of reduction of cytochrome *c* by 75%. To determine whether this block affected *bd* oxidase or the bc_1 complex, we collected NADH reduced - air oxidized absorbance spectra (Fig. 2C) in the presence of trifluoperazine. Following incubation with 100μM trifluoperazine, the peaks corresponding to cytochromes -*b*, -*c*, -*a*, and -*d* not only failed to increase, but were oxidized below baseline. Thus, phenothiazines inhibit electron transport in *MTb* at a point prior to the step involving cytochrome *c*.

Example 10

[00322] To test whether phenothiazines acted upon NADH:menaquinone oxidoreductase activity, the electron transport chain was blocked with 10 mM KCN, and the rate of oxidation of NADH in the presence of menaquinone-1 was measured. The addition of Compound 1 resulted in a concentration dependent decrease in NADH:menaquinone oxidoreductase activity with an IC_{50} of 158 μM (Figure 3A). Flavone, a weak inhibitor of type II NADH oxidoreductase, suppressed NADH oxidation with an IC_{50} of 750 μM. Classic inhibitors of type I NADH oxidoreductase, namely rotenone (10 μM), piericidin A (10 μM), and pyridaben (10 μM) did not inhibit *MTb* NADH:menaquinone oxidoreductase activity. Additionally, deamino-NADH, a type I NADH:menaquinone oxidoreductase specific substrate, was not oxidized by *MTb* membranes. Therefore, type II NADH oxidoreductase activity is a specific target site of phenothiazines, and appears to be dominant in *MTb* membranes grown under aerobic conditions.

[00323] We next tested whether phenothiazines inhibited succinate dehydrogenase (complex II). In this assay, the dye dichlorophenolindophenol (DCIP) was used as an electron acceptor and succinate as the electron donor. Phenothiazine concentrations as high as 400 μM did not inhibit the reduction of dye. This was confirmed using the amperometric assay with succinate as the electron donor. The rate of oxygen consumption in this case was not altered by the

phenothiazines. The activity of phenothiazines is therefore specific for type II NADH:menaquinone oxidoreductase, and not for complex II.

[00324] To further investigate this class of drugs, a series of phenothiazines were tested in an *M. tuberculosis* growth inhibition assay. A number of analogues of chlorpromazine (Figure 3B) (GlaxoSmithkline Pharmaceuticals, Philadelphia, PA) were initially screened for inhibition of *M. smegmatis* growth. The MICs for $H_{37}Rv$ of Compound 1 (1.11 μg/ml), Compound 2 (3.44 μg/ml), Compound 3 (3.96 μg/ml), TPZ (19.2 μg/ml), CPZ (9.23 μg/ml), rifampicin (0.5 μg/ml) and isoniazid (0.15 μg/ml) were determined using the Bactec 960 system. The bacteriocidal effect of these drugs was demonstrated by the failure to recover colonies on plates from cultures at 35 days. Phenothiazines that were ineffective inhibitors of *M. smegmatis* growth were equally poor inhibitors of *MTb* growth in the Bactec 960 system.

[00325] The anti-mycobacterial activity of phenothiazines has been sporadically reported in the literature over the past 40 years. Ratnakar and coworkers tested six commercially available phenothiazines against H37Rv, and while all six agents displayed some antibacterial activity, trifluoperazine proved to be the most potent. Phenothiazines exert tuberculocidal activity *in vitro* against *Mycobacterium tuberculosis* strains resistant to isoniazid, rifampin, streptomycin, pyrazinamide and ethambutol. Trifluoperazine is effective against virulent *MTb* strain H37Rv in a macrophage model of infection, and is reported as synergistic with both isoniazid and rifampicin[25, 26]. It has been suggested[21] that the mechanism of action of phenothiazines is related to the antagonism of a calmodulin-like protein. However, a search of the *MTb* genome failed to identify a calmodulin homologue.

[00326] As discussed above, the *in vitro* concentration required for bacteriocidal activity is more than 100 fold greater than the clinical concentration of drug *in vivo*[26]. Our data indicates that Compound 1 is 12-fold more potent than trifluoperazine in the Bactec 960 growth assay. Phenothiazines are reported to be concentrated within macrophages, which may significantly reduce the required dose for treatment[1]. In contrast to trifluoperazine, Compound 1 is water soluble at pH 7.4, and cellular toxicity studies of this compound in A-537 fibroblasts indicates an ID_{50} of more than 128 μg/ml (data not shown). With these favorable qualities Compound 1 represents an important new compound for the treatment of tuberculosis.

Example 11

[00327] Compounds

[00328] The compounds of the invention can be readily prepared by the skilled artisan according to routine synthetic methods such as those described in Kemp et al., Organic Chemistry, Worth Publishers, Inc., New York, 1980 and March, Advanced Organic Chemistry, 4th ed., John Wiley & Sons, Inc., New York, 1992, each of which is incorporated herein by reference in its entirety.

[00329] Many phenothiazine compounds and analogs thereof are commercially available. For example, trifluoperazine and chlorpromazine are available from Sigma-Aldrich, Inc. (product numbers: 28,388-6 and 26060, respectively). Also 2-(trifluoromethyl)phenothiazine, 2-methylthiophenothiazine, promazine, propionylpromazine, ethopropazine, thioridazine, propericyazine, acetopromazine, perphenazine, 10-(2-methyl-allyl)-10h-phenothiazine, 10-(1-ethyl-3-pyrrolidinylmethyl)-phenothiazine, 7-nitro-2-(trifluoromethyl)-phenothiazine and 2-chloro-10-(2-cyanoethyl)-10h-phenothiazine are available from Sigma-Aldrich, Inc.

Sequences

[00330] The following listed sequences are referred to throughout the specification. The amino acid sequences are presented in the amino to carboxy direction, from left to right. The amino and carboxy groups are not presented in the sequence. The nucleotide sequences are presented by single strand only, in the 5' to 3' direction, from left to right. Nucleotides and amino acids are represented in the manner recommended by the IUPAC-IUB Biochemical Nomenclature Commission or (for amino acids) by three letters code.

```
>M. tuberculosis H37Rv|Rv1854c|Ndh: 463 aa - PROBABLE NADH
DEHYDROGENASE NDH  (SEQ ID NO:1)
MSPQQEPTAQPPRRHRVVIIGSGFGGLNAAKKLKRADVDIKLIARTTHHLFQPLLYQVAT
GIISEGEIAPPTRVVLRKQRNVQVLLGNVTHIDLAGQCVVSELLGHTYQTPYDSLIVAAG
AGQSYFGNDHFAEFAPGMKSIDDALELRGRILSAFEQAERSSDPERRAKLLTFTVVGAGP
TGVEMAGQIAELAEHTLKGAFRHIDSTKARVILLDAAPAVLPPMGAKLGQRAAARLQKLG
VEIQLGAMVTDVDRNGITVKDSDGTVRRIESACKVWSAGVSASRLGRDLAEQSRVELDRA
GRVQVLPDLSIPGYPNVFVVGDMAAVEGVPGVAQGAIQGAKYVASTIKAELAGANPAERE
PFQYFDKGSMATVSRFSAVAKIGPVEFSGFIAWLIWLVLHLAYLIGFKTKITTLLSWTVT
FLSTRRGQLTITDQQAFARTRLEQLAELAAEAQGSAASAKVAS
```

```
>M. tuberculosis H37Rv|Rv1854c|ndh: 1392 bp - PROBABLE NADH
DEHYDROGENASE NDH (SEQ ID NO:2)
atgagtccccagcaagaacccacagcgcaaccacctcgtaggcatcgagttgtgatcatc
ggatctgggttcggcgggctaaacgcggcaaagaagctcaagcgggccgacgttgacatc
```

aagctgatcgcgcgcaccacccatcacctgttccagccgctgctgtaccaagtggccacc
gggattatctccgagggagaaatcgctccgccgacccgggtcgtgctgcgtaagcagcgc
aatgtccaggtactgttgggcaacgtcacccacatcgacctggccgggcagtgcgtcgtc
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gctggccagtcttatttcggcaacgaccatttcgccgaattcgcacccggcatgaagtcc
atcgacgacgcgttggagttgcgtggccgcatattgagcgctttcgagcaagccgaacgg
tccagcgatccggaacggcgggccaagctactgacattcaccgttgtcggggctggcccc
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ttcctcagtactcgccgcggccagctgaccatcaccgaccagcaggcatttgcgcgaacg
cggctcgaacagctggccgagctggccgccgaggcgcagggctcagcggcaagcgctaag
gtggccagctag

>M. tuberculosis H37Rv|Rv0392c|NdhA: 470 aa - PROBABLE
MEMBRANE NADH DEHYDROGENASE NDHA; (SEQ ID NO:3)
MTLSSGEPSAVGGRHRVVIIGSGFGGLNAAKALKRADVDITLISKTTTHLFQPLLYQVAT
GILSEGDIAPTTRLILRRQKNVRVLLGEVNAIDLKAQTVTSKLMDMTTVTPYDSLIVAAG
AQQSYFGNDEFATFAPGMKTIDDALELRGRILGAFEAAEVSTDHAERERRLTFVVVGAGP
TGVEVAGQIVELAERTLAGAFRTITPSECRVILLDAAPAVLPPMGPKLGLKAQRRLEKMD
VEVQLNAMVTAVDYKGITIKEKDGGERRIECACKVWAAGVAASPLGKMIAEGSDGTEIDR
AGRVIVEPDLTVKGHPNVFVVGDLMFVPGVPGVAQGAIQGARYATTVIKHMVKGNDDPAN
RKPFHYFNKGSMATISRHSAVAQVGKLEFAGYFAWLAWLVLHLVYLVGYRNRIAALFAWG
ISFMGRARGQMAITSQMIYARLVMTLMEQQAQGALAAAEQAEHAEQEAAG

>M. tuberculosis H37Rv|Rv0392c|ndhA: 1413 bp - PROBABLE
MEMBRANE NADH DEHYDROGENASE NDHA (SEQ ID NO:4)
atgacgctctcatctggtgaaccctcggccgtcggcgggcgccatcgcgtggtcatcatc
ggtagtggattcggcggcctgaatgcggccaaggcgcttaaacgggcggatgtcgacatc
acgctgatctccaagacaacgacccacctgttccagccgctgctgtatcaagtggccacc
gggatcttgtccgagggcgacattgccccgaccacccggctgatcctgcgccggcaaaag
aacgtccgggtgttgctgggcgaggtcaacgcgatcgacctgaaagcgcagacggtcacg
tcgaaattgatggacatgaccacggtgacgccgtacgacagcctcatcgtggccgccggc
gcacagcagtcctacttcggcaacgacgaattcgccaccttcgcgcccggaatgaagacc
atcgacgacgcgctggagctgcgcggccgcatcctgggcgcgttcgaggccgccgaggtc
agcaccgaccatgccgaacgggagcggcgcctgacgttcgtcgtcgtcggcgctgggccg
accggcgtcgaggtggctgggcagatcgtcgagctcgccgagcgcaccctggcaggcgcg
tttaggaccatcacgcccagtgagtgccgggtgatcctgctcgacgccgcacccgcggtg
ttgccgccgatgggtccaaagctgggtctcaaggcacaacggcggctggaaaagatggac
gtcgaggttcaactcaacgcgatggtgaccgcggtcgactacaaaggcatcaccatcaag

gaaaaggacggcggcgaacgccgcatcgaatgcgcgtgcaaggtttgggcggccggcgtg
gcggccagcccgctgggcaagatgatcgccgagggatccgacggaaccgaaatcgaccgg
gccggaagggtgatcgtggaacccgatctcaccgtcaagggacatccgaacgtcttcgta
gtcggcgatctgatgttcgtgcccggcgtacccggggtggctcagggcgcgatccagggg
gcccgatacgccaccacggtgatcaaacacatggtcaagggcaatgacgacccagccaat
cgcaagccgttccattacttcaacaagggcagcatggcgacgatctcccgccacagcgcc
gtcgcgcaggtcggcaagctggagtttgccgggtacttcgcctggctggcgtggctggtg
ctgcacctggtctacctggtcggctatcggaaccgcatcgcagccctgttcgcctgggg
atctccttcatggccgcgcccgcggccagatggccatcaccagccagatgatctacgcc
aggttagtgatgaccttgatggaacagcaggcacaaggagcgctggcagccgccgaacag
gccgagcacgccgagcaagaggcagcgggttag

Menaquinone methyltransferase; M. tuberculosis CDC1551; gene acc no. NC 0022755 (SEQ ID NO:6); protein acc no. NP_334993 (SEQ ID NO:5).

Flavin adenine dinucleotide dehydrogenase - (also designated "flavin binding family"); M. tuberculosis CD1551; gene acc no. NC 0022755 (SEQ ID NO:8); protein acc no. NP 335000 (SEQ ID NO:7).

Fumarate reductase - also M. tuberculosis H37Rv - gene acc. no. Z74020 (SEQ ID NO:10); protein acc no. CAA 98311 (SEQ ID NO:9)

Nitrate reductase - in M. tuberculosis H37Rv - gene acc. no. Z95584 (SEQ ID NO:12); protein acc. no. CAB 09020 (SEQ ID NO:11).

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[00331] The references listed below as well as all references cited in the specification are incorporated herein by reference to the extent that they supplement, explain, provide a background for or teach methodology, techniques and/or compositions employed herein.

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